Université de Montréal

The kallikrein-kinin system in relation to retinal vessel tone in the streptozotocin-diabetic rat model

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Ce mémoire intitulé:

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SOMMAIRE

Environ 3% de la population mondiale dont 1.5 millions de canadiens souffrent du diabète. La morbidité associée au diabète de type I ou II (diabète insulino-dépendant ou non-insulino-dépendant), résulte de complications sérieuses, telles que; microangiopathie, néphropathie, neuropathie et rétinopathie. La prevalence de tous les types de rétinopathie dans la population diabétique augmente avec la durée du diabète et l'âge du patient.

Des données soutenant la participation du système kallikreine-kinine dans la pathogenèse du diabète a été obtenue à partir de patients diabétiques et de modèles animaux de diabète par plusieurs groupes. Les kinines sont des agents vasoactifs efficaces dans la microcirculation, où elles produisant les effets par des récepteurs sur le muscle lisse des microvaissaux ou des cellules endothéliales suite à une réaction inflammatoire ou un dommage tissulaire.

Des données de la littérature ont démontré que le système kallikreine-kinine est exprimé au niveau des yeux de lapins et de porcs. Aussi, à l'aide d'analyses RT-PCR et Southern blot, l'expression et la localisation des differentes composantes de ce système tels que la kallikreine tissulaire, le kininogene de faible poids moléculaire et les récepteusrs B_1 et récepteurs B_2 à forte densité ont été identifiées dans la rétine humaine. En outre, l'hybridation in situ a identifié la localisation cellulaire des ARN messagers des composantes de ce système dans les tissus oculaires et dans les

cellules endothéliales des vaisseaux sanguins oculaires humains. Cependant, il n'y a aucune étude sur la fonction physiologique du système kallikreine-kinine et son rôle potentiel dans le développement de la rétinopathie diabétique au niveau de l'oeil. Le but de cette étude était d'étudier le rôle des kinines dans la régulation du tonus vasculaire rétinien, avec trois objectifs spécifiques:

..i) déterminer la nature des récepteurs aux kinines impliqués en utilisant des agonistes et antagonistes selectifs,

ii) investiguer les voies de signalisation impliquées dans l'effet vasoactif des kinines dans la rétine, et

iii) étudier l'effet de la bradykinine (BK) et de la des-Arg^9-BK sur le tonus des vaisseaux rétiniens chez le rat adult Wistar diabétique suite à l'injection de streptozotocine (STZ).

Les résultats de cette étude montrent que:

i) la BK dilate les vaisseaux rétiniens chez les rats contrôles et diabétique, et l'effet vasodilatateur de la BK passe par la stimulation des récepteurs B_2,

ii) la des-Arg^9-BK ne dilate pas les vaisseaux rétiniens chez le rat contrôle,

iii) la des-Arg^9-BK dilate les vaisseaux rétiniens chez les rats diabétiques et l'effet vasodilatateur de la des-Arg^9-BK passe par la stimulation des récepteurs B_1,

iv) l'effet vasodilatateur des kinins implique une G-proteins (G_i/G_o),

v) la libération de l'oxyde nitrique et l'augmentation du calcium intracellulaire par l'influx à partir du milieu extracellulaire ne sont pas impliqués dans les effets de la BK et la des-Arg^9-BK. Mais,

vi) l'augmentation de Ca^{2+} intracellulaire à partir des réserves intracellulaires sensible et insensible à l'IP_3 et la libération de prostaglandine I_2 (prostacycline; PGI_2), métabolite de la voie de la cyclooxygénase, sont impliquées dans les effets de la BK et la des-Arg^9-BK.

Nos résultats fournissent pour la première fois des évidences fonctionnelles directes que le système kallikreine-kinine joue un rôle crucial dans la régulation du tonus vasculaire rétinien et que ce système peut avoir un rôle principal durant les premières étapes de développement de la rétinopathie diabétique.

Mots clés: récepteurs B_2, récepteurs B_1, rétinopathie diabétique, vasodilatation.

SUMMARY

About 3% of the world population including 1.5 million Canadians suffer from diabetes. The morbidity associated with long-standing diabetes of either type I or II (insulin-dependent or non-insulin-dependent diabetes mellitus respectively) results from a number of serious complications, namely microangiopathy, nephropathy, neuropathy and retinopathy. The prevalence of retinopathy in the diabetic population increases with the duration of diabetes and is directly correlated with patient age.

The true cause of diabetic microvascular disease is unknown. There is increasing evidence, from diabetic animal models and diabetic patients, which supports the involvement of tissue kallikrein in diabetes mellitus. Kinins are potent vasoactive agents in the microcirculation, producing effects through receptors on microvessel smooth muscle or endothelial cells following inflammatory insult or tissue damage.

Previous studies have demonstrated that kallikrein-like enzymatic activity exists in tissue homogenates of rabbit and swine eyes. Furthermore, it has been shown by molecular studies that mRNAs for key components of the kallikrein-kinin system, including tissue kallikrein and low molecular weight kininogen, are present in human ocular tissues. In addition, high levels of kinin B_1 and B_2 receptors have been detected in human retina. Also, *in situ* hybridization has identified mRNAs for the components of the kallikrein-kinin system in ocular tissues and in endothelial cells of human ocular blood vessels. However, there has been no study on the physiologic

function of the kallikrein-kinin system in ocular tissues and its potential roles in the development of diabetic retinopathy.

The aim of the current study was to investigate the effect of kinins on retinal vessel tone, with three specific objectives:

i) to establish which receptors mediate the vascular response to kinins using selective agonists and antagonists,

ii) to determine which signal transduction pathways mediate the vascular effects of kinins in the retina of healthy adult male Wistar rats, and

iii) to study the effect of Bradykinin (BK) and des-Arg^9-BK on retinal vessel tone in the adult streptozotocin (STZ)-diabetic rat.

The present study shows that:

i) BK dilates retinal vessels in control and STZ-diabetic rats,

ii) the vasodilator effect of BK is mediated by stimulation of B_2 receptors,

iii) des-Arg^9-BK is without effect on retinal vessel tone in control rats, but des-Arg^9-BK dilates retinal vessels in STZ-diabetic rats,

iv) the vasodilator effect of des-Arg^9-BK is mediated by stimulation of B_1 receptors,

v) downstream of B_1 or B_2 receptors, the vasodilator effect of kinins is transduced by G_i/G_o-proteins, and

vi) the release of nitric oxide (NO) or the influx of extracellular Ca^{2+} are not involved in the vasodilation evoked by either BK or des-Arg^9-BK. In contrast, libera-

tion of Ca^{2+} from both intracellular, IP_3-sensitive and non-IP_3-sensitive, pools are involved as well as the release of prostaglandin I_2 (prostacyclin; PGI_2).

This study is the first to provide direct functional evidence that the kallikrein-kinin system may play a role in the retinal circulation, and may be a factor early in the pathological development of diabetic retinopathy.

Key words: B_2 receptors, B_1 receptors, diabetic retinopathy, vasodilation.

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LIST OF ABBREVIATIONS

AA	Arachidonic Acid
AC	Adenylate Cyclase
ACE	Angiotensin-I-Converting Enzyme
ATP	Adenosine Triphosphate
BHQ	2,5-Di-tert-butylhydroquinone
BK	Bradykinin
$[Ca^{2+}]_i$	Intracellular Calcium
cADP-ribose	Cyclic ADP-ribose
cAMP	Cyclic Adenosine Monophosphate
cGMP	Cyclic Guanosine Monophosphate
$cPLA_2$	Phospholipase A_2
COX-2	Cyclooxygenase-2
des-Arg^9-BK	des-Arg^9-Bradykinin
des-Arg^{10}-Hoe140	des-Arg^9-D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-Bradykinin
DG	Diacylglycerol
eNOS	endothelial Nitric Oxide Synthetase
GC	Guanylyl Cyclase
$GdCl_3$	Gadolinium Chloride
GP	G-Protein
HMWK	High Molecular Weight Kininogen
Hoe-140	D-Arg-[Hyp^3, Thi^5,$_D$-Tic^7, Oic^8]-BK
IDDM	Insulin Dependent Diabetes Mellitus
IL-1β	Interleukin-1β
iNOS	Inducible Nitric Oxide Synthetase
IP_3	Inositol Triphosphate
KD	Kallidin
LMWK	Low Molecular Weight Kininogen
L-NAME	Nω-Nitro-L-Arginine Methyl Ester
MAP kinase	Mitogen Activated Protein Kinase
Mins	Minutes
NF-κB	Nuclear Factor Kappa B

NIDDM	Non-Insulin Dependent Diabetes Mellitus
NO	Nitric Oxide
O_2	Oxygen
PC-PLC	Phosphatidylcholine specific Phospholipase C
PDGF	Platelet Derived Growth Factor
PGI_2	Prostacyclin
PIP_2	Phosphatidylinositol 4,5-diphosphate
PKC	Protein Kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
R	Membrane Receptor
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
RyR	Ryanodine Receptor
sGC	Soluble Guanylate Cyclase
STZ	Streptozotocin
TNFα	Tumor Necrosis Factor-α
TPC	Trans-2-phenylcyclopropylamine
U-46619	9,11-dideoxy-9a,11 α–methanoepoxyprostaglandin $F_{2\alpha}$
VEGF	Vascular Endothelial Growth Factor

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CHAPTER I

1. INTRODUCTION

1.1. DIABETES MELLITUS

Diabetes mellitus is a chronic disorder of carbohydrate, fat and protein metabolism. About 3% of the world population, including 1.5 million Canadians, suffer from diabetes, making it one of the most common noncommunicable diseases [1]. This disease can be categorised into two common variants (Type I and Type II) which differ in their pattern of inheritance, responses to insulin, origins, and less commonly in specific genetic defects of β-cell function (Table I) [2] & (Table II) [1]. Type I diabetes, also called IDDM, and previously referred to as juvenile onset diabetes, accounts for about 10 % of all cases of primary diabetes. This is mainly due to a defect in insulin secretion. Most patients (80%-90%) have type II diabetes, also termed NIDDM and previously referred to as adult onset diabetes. Its onset follows a defect in the response to insulin due to either a non responsive insulin receptor or to a defect in the transduction machinary [1].

It should be stressed that while the two catagories of diabetes have different pathologic mechanisms, the long-term complications produced in blood vessels, kidneys, nerves, and eyes are the same. Such complications are the major causes of morbidity and finally death from diabetes [1].

TABLE I. CATEGORIES OF DIABETES MELLITUS

Primary Diabetes
- Type I (insulin-dependent diabetes mellitus, IDDM)
- Type II (non-insulin-dependent diabetes mellitus, NIDDM)
- Genetic defects of β-cell function (including maturity-onset diabetes of the young [MODY])

Secondary Diabetes
- Infections (e.g.,congenital rubella)
- Endocrinopathies (e.g., pituitary tumours)
- Drugs (e.g.,corticosteroids)
- Other genetic disorder (e.g., Down's syndrome)
- Gestational diabetes mellitus

Modified from the report of the Executive Committee on the Diagnosis and Classification of Diabetes Mellitus: Diabetic Care 20: 1183-1197, 1997 [2].

TABLE II. COMPARISON OF TYPE I VS TYPE II DIABETES MELLITUS

	TYPE I (IDDM)	TYPE II (NIDDM)
Clinical	Onset <20 yr Decreased blood insulin	Onset >30 yr Normal or increased blood insulin
Pathogenesis	Autoimmunity Severe insulin deficiency	Insulin resistance Relative insulin deficiency

Modified from pathologic basis of disease. W.B. Saunders Company. Philadelphia. 913-929, 1999 [1].

1.1.1. TYPE I DIABETES MELLITUS

This form of diabetes results from a severe drop in the level of circulating insulin due to a reduction in β-cell mass. Three interrelated mechanisms are responsible for islet cell destruction: genetic susceptibility, autoimmunity, and an environmental insult [1].

1.1.2. TYPE II DIABETES MELLITUS

Pathogenesis of type II diabetes mellitus remains enigmatic. Genetic factors are even more important in type II than in type I diabetes. The two metabolic defects that characterize type II diabetes are: a derangement in β-cell secretion of insulin and a decreased response of peripheral tissues to insulin (insulin resistance) [1].

1.1.3. PATHOLOGY OF DIABETES

The morbidity associated with long-standing diabetes of both types results from a number of serious complications, namely microangiopathy, nephropathy, neuropathy and retinopathy [1]. There is extreme variability among patients with regards to the time of onset of these complications, their severity, and the particular organ(s) that are involved. In those whom are able to control well their diabetes, the onset may be delayed [1]. The cause of diabetic microvascular disease has not been established [3]. The overproduction of cytokines including IL-1β and TNFα following an autoimmune response leads to the destruction of pancreatic β-cells and consequently the induction of diabetes mellitus type I [4]. Furthermore, most of the available experimental and clinical evidence suggests that the complications are a consequence of the metabolic derangement, in particular the hyperglycemia [1,3]. The oxidative stress following hyperglycemia, may activate NF-κB which induces kinin B_1 receptors. The latter is generally under-expressed in normal conditions but it is highly inducible by

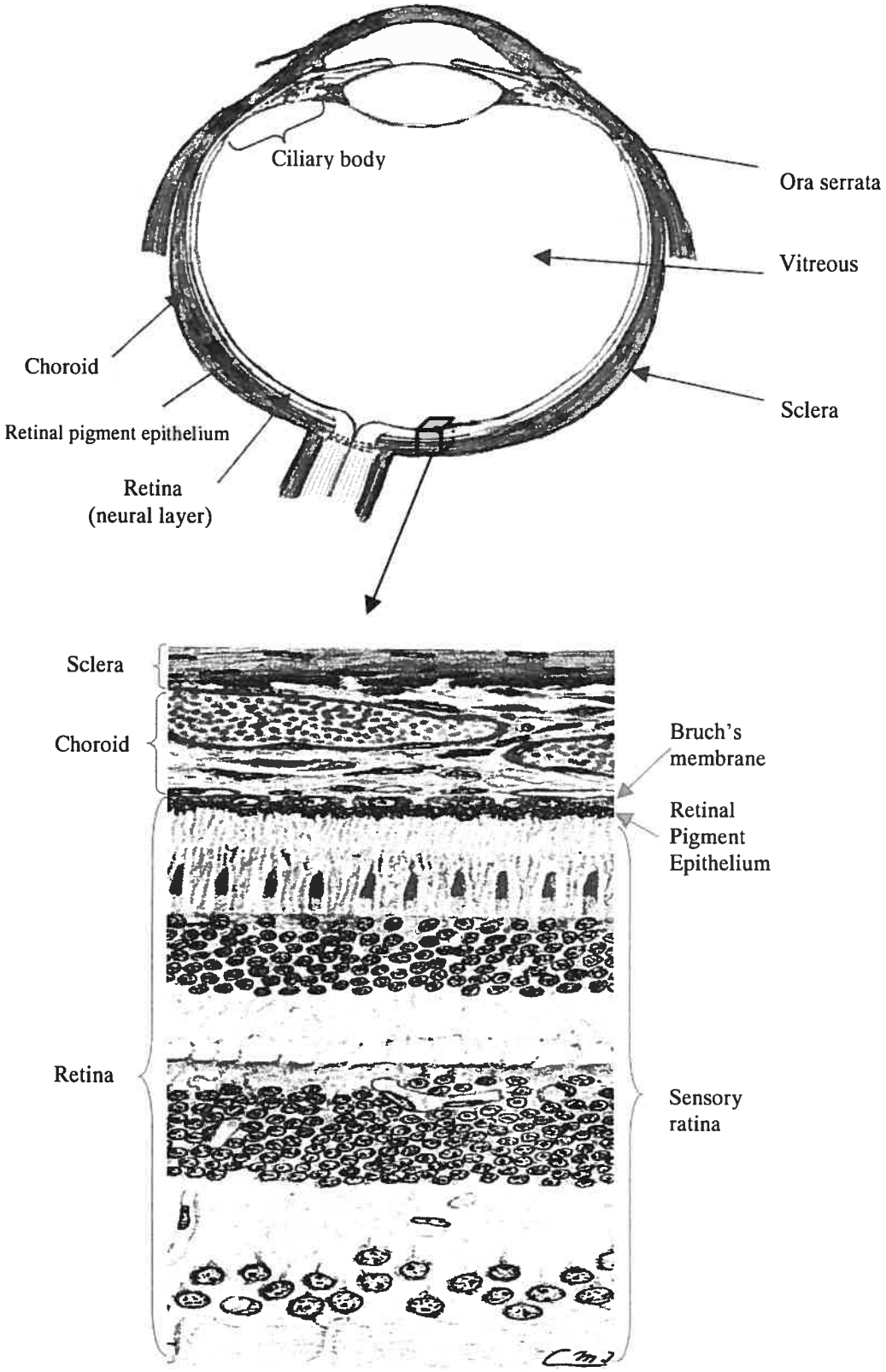
inflammatory mediators [5]. In addition, there is evidence to support the involvement of the kallikrein-kinin system in diabetes mellitus [6]. Both the overproduction of cytokines and hyperglycemia stimulate the kallikrein-kinin system which is known to promote arteriolar vasodilation [7].

1.2. THE RETINA AND ITS BLOOD VESSELS

The retina is a thin, multilayered sheet of neural tissue that lines the inner aspect of the posterior two-thirds of the wall of the ocular globe. It extends almost as far anteriorly as the ciliary body, ending at that point in a ragged edge termed the ora serrata. The outer surface of the sensory retina is apposed to the retinal pigment epithelium and thus associated with Bruch's membrane, the choroid, and the sclera (Fig. 1) [8,9]. The inner surface of the retina is in contact with the vitreous [9].

The retina receives its blood supply from two sources: the choriocapillaris immediately outside of Bruch's membrane, which supplies the outer third of the retina; and branches of the central retinal artery, which supply the inner two-thirds (Fig. 2). The capillaries of the retina vary in diameter from about 3 to 6 μm and they are surrounded by a thick basal lamina, pericytes, and endothelial cells. The retinal blood vessels have a non-fenestrated endothelium as do cerebral blood vessels. Such an endothelial anatomy allows only extremely small molecules such as O_2 and CO_2 to pass into or out of the retina. It forms part of the blood-retinal barrier and regulates the selective exchange of material and cells between the retina and the blood [9] . In

contrast, in other tissues, the clefts between the capillary endothelial cells are wide open, therefore almost all dissolved substances of the plasma can pass from the blood into the tissues [9]. The blood-retinal barrier in its entirety consists of two anatomic characteristics: 1) tight junctions between the endothelial cells of the retinal vessels, 2) tight junctions between the retinal pigment epithelial cells [3]. The blood-retinal barrier serves to maintain the proper ionic balance required for retinal function [3,9]. The retina has the highest demand for O_2 per gram tissue in the body. A marked drop in the supply of O_2 to the retina can result in ischemia and the pathological manifestations of disease such as diabetic retinopathy [9].



FIE 1. The retinal circulation in relation to the choroid and the sclera. See text for information [8].

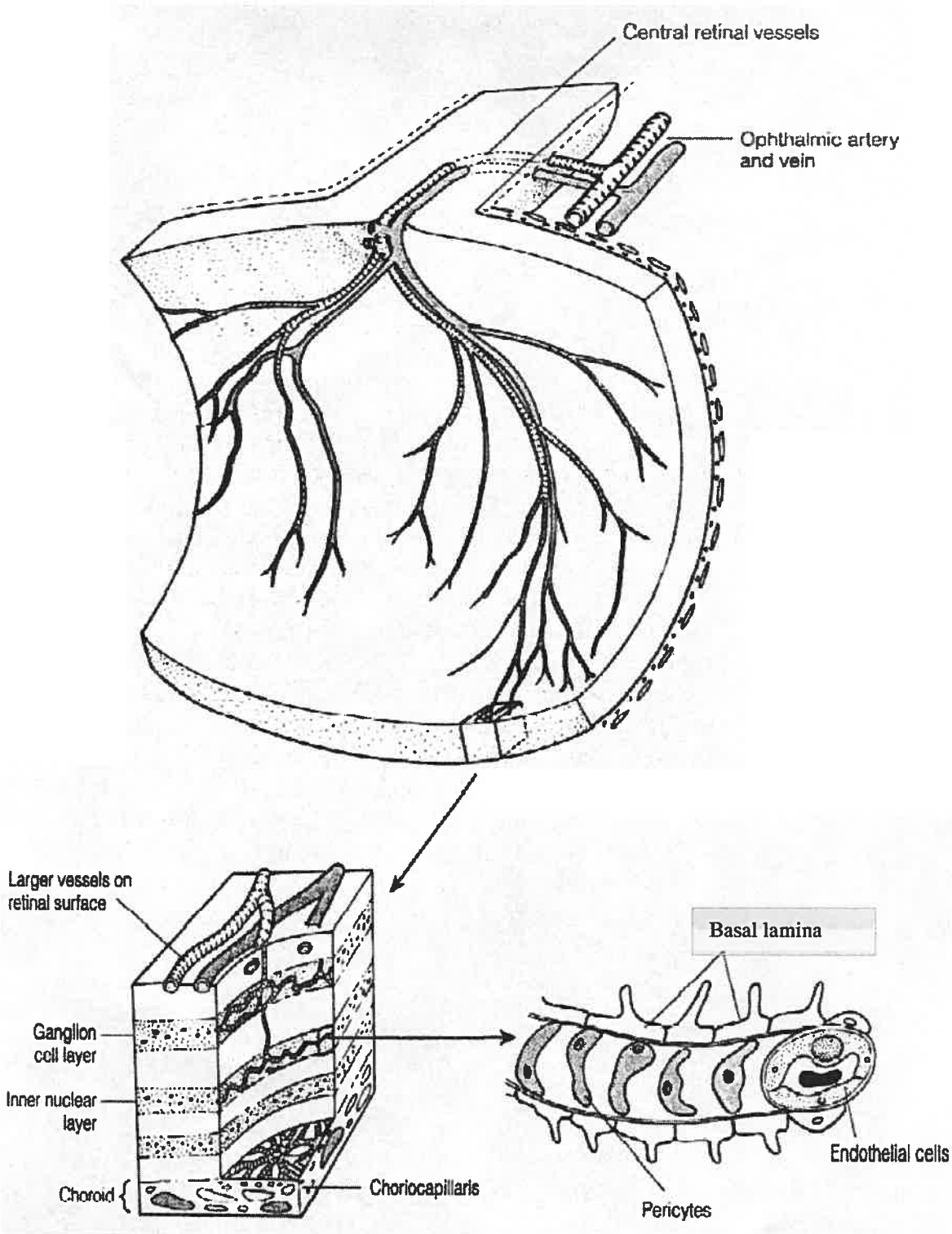


FIGURE 2. The retina and its blood vessels (the branches from the choriocapillaries and central retinal artery). In right hand, the structure of the retinal capillaries, from outside: basal lamina, pericytes, and endothelial cells [9].

1.3. OCULAR DIABETIC COMPLICATIONS

1.3.1. CLASSIFICATION AND INCIDENCE OF DIABETIC RETINOPATHY

Visual impairment leading to total blindness is among the most feared consequences of long-standing diabetes [1,3]. This disease is currently the fourth leading cause of acquired blindness in Western countries [1,3]. It is estimated that if a patient is diagnosed as a diabetic at age 30, there is a 10% chance he will have some degree of diabetic retinopathy by age 37 and up to 90% chance by age 55 [1]. The ocular involvement may take the form of cataract formation, glaucoma or retinopathy [1]. The prevalence of all types of retinopathy in the diabetic population increases with the duration of diabetes and patient age [3]. With advances in therapy, the life span of diabetic patients has improved, but the prevalence of secondary retinal disease has greatly increased. Approximately 60% of diabetic patients develop retinopathy 15 to 20 years after their original diagnosis. Furthermore, about 2% of the diabetic population have visual impairment, severe enough to be considered legally blind, that is attributable to retinopathy [10]. There are two forms of diabetic retinopathy known as nonproliferative and proliferative retinopathy [11].

1.3.2. NONPROLIFERATIVE DIABETIC RETINOPATHY

Early in the course of diabetic retinopathy, certain physiologic abnormalities are seen. These include impaired autoregulation of the retinal vasculature, alterations in retinal blood flow, and breakdown of the blood-retinal barrier [3]. Retinal microvascular changes that occur in nonproliferative retinopathy are limited to the confines of the retina and do not extend beyond the retinal limiting membrane [3]. Characteristic findings include dilation of veins, microaneurysms, intraretinal hemorrhages, diffuse edema, and focal exudates [3]. The nonperfusion following impaired autoregulation of the retinal vasculature produces hypoxia which can later lead to proliferative retinopathy [3,10].

1.3.3. PROLIFERATIVE DIABETIC RETINOPATHY

The proliferative stage of diabetic retinopathy is subdivided into a proliferative phase (neovascularization) and a contraction (cicatricial) phase [3,10]. The neovascularization occurs in response to severe ischemia and hypoxia of the retina [3,10]. The new capillaries contain both endothelial cells and pericytes but are incompletely formed and poorly supported [10].

1.3.4. VASODILATION IN DIABETIC RETINOPATHY

Changes in vascular flow and caliber begin very early after injury and develop at varying rates, depending on the severity of the injury [1]. In the retinal circulation, pericytes play a contractile function. Thus, pericyte degeneration in early stages of diabetic retinopathy as a consequence of diabetes leads to vasodilation [10]. Furthermore, retinal vessel damage due to diabetes stimulates the overproduction of cytokines derived from lymphocytes and macrophages, and activation of NF-κB or the release of vasodilator agents which promote arteriolar vasodilation [4,5]. The vasodilation is associated with retinal ischemia. It is perhaps an attempt by the retinal circulation to releave the ischemia through vasodilation leading to increased blood flow.

1.3.5. RETINOPATHY IN STZ-DIABETIC RAT MODEL

Ocular complications following diabetes have been reported in a variety of laboratory species [12-14]. In animal models as in human, the development of diabetic retinopathy depends on the duration and severity of hyperglycemia. The rat as a model of diabetic retinopathy offers advantages over other large animals in terms of cost and housing requirements and relatively rapid onset of significant anatomic criteria such as capillary cell loss. Although, because of the short life span of rats all changes that occur in the human retina due to diabetes do not appear in the rat, but the early stages of diabetic retinopathy such as blood-retinal barrier breakdown and

degenerative changes in pericytes have been demonstrated in the diabetic rat and have been partly ascribed to VEGF [14,15]. The vasodilator agents which mediate early vasodilation have not been established

1.4. THE KALLIKREIN-KININ SYSTEM

The kallikrein-kinin system includes the kinins, their precursors (HMWK, LMWK, and T-kininogen), and their relative enzymes including plasma and tissue kallikreins, T-kininogenase, kininase I, kininase II [16-23].

1.4.1. KININ BIOSYNTHESIS

Kinins are potent (subnanomolar K_d), short-lived (30 seconds or less) mediators. They belong to a small family of structurally related 9-11 amino acid peptides including BK, kallidin (KD; Lys-BK), T-kinin (Ile-Ser-BK; exclusively in rats), and des-Arg-kinins (Table III) [16-26].

TABLE III. PRIMARY STRUCTURE OF MAMMALIAN KININS

	1 2 3 4 5 6 7 8 9
Bradykinin (BK)	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH
Kallidin	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH
T-Kinin	Ile-Ser-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH
des-Arg^9-BK	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-OH

Modified from Brain kallikrein-kinin system: from receptors to neuronal pathways and physiological functions. Handbook of chemical neuroanatomy. 16: peptide receptors, part I. 241-300 [16].

BK and KD are generated following the proteolytic cleavage of their respective precursors, HMWK and LMWK by plasma and tissue kallikreins [22,23] (Fig. 3). A single gene codes for plasma kallikrein, whereas tissue kallikrein is a member of a multigene family that shows different patterns of tissue-specific gene expression [22]. T-kinin was identified exclusively in the rat [24,25] and could be generated from T-kininogen under the enzymatic action of T-kininogenase [26]. Kinins stimulate B_1 or B_2 receptors on the cell surface to produce their biological effects [21,22].

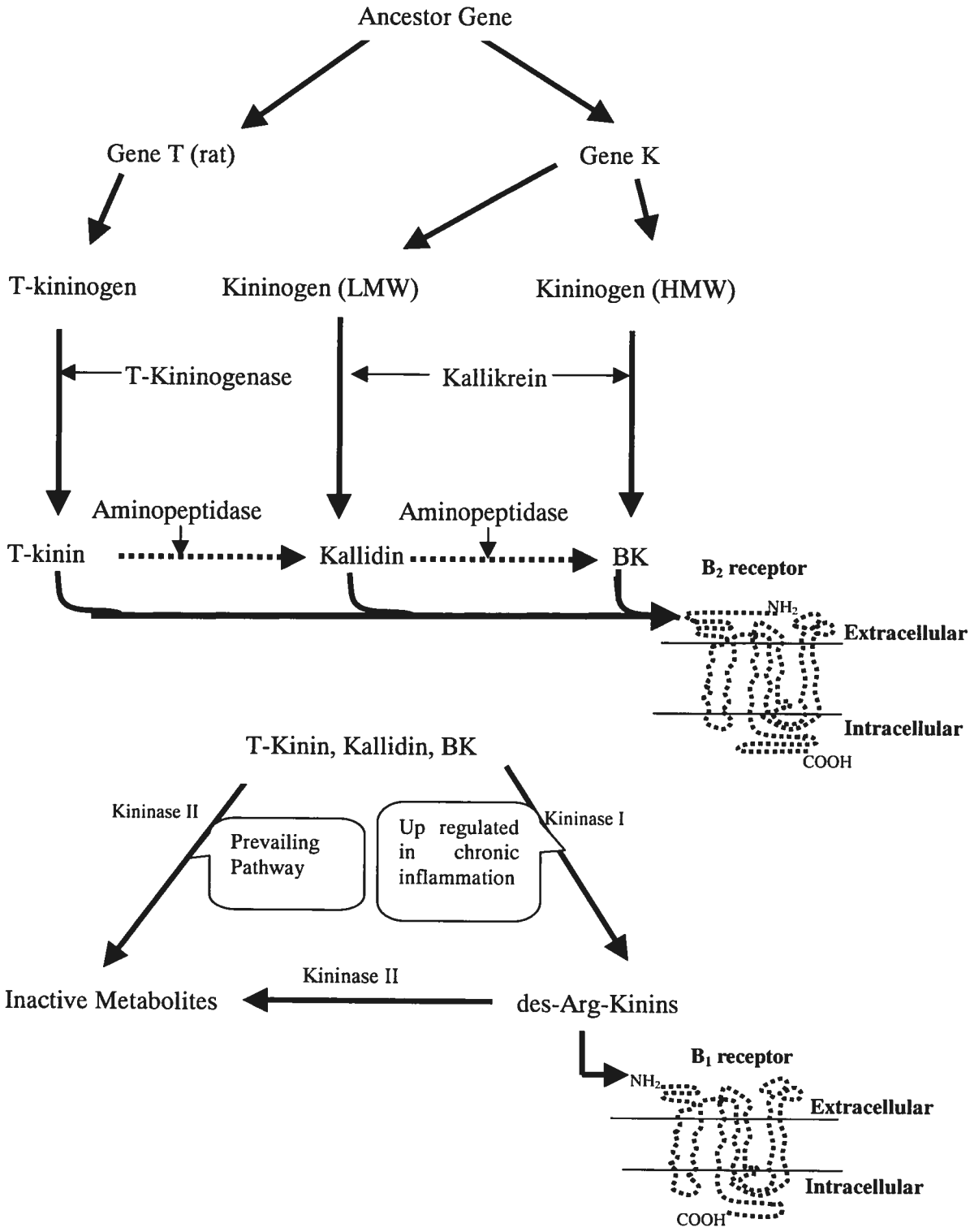
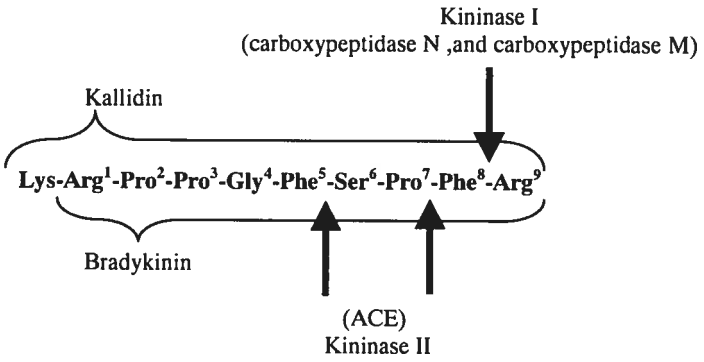


FIGURE 3. Kinin formation and inactivation. All the components of the kallikrein-kinin system are shown in this diagram. The B_1 receptor is not readily expressed under normal physiological condition, it can be upregulated during chronic pathological states (e.g. inflammation, hyperthermia, diabetes and epilepsy) [16]. See text for details.

1.4.2. KININ METABOLISM

Metabolic degradation is an important mechanism by which kinin action is terminated. Kinins are broken down by a group of amino- , carboxy- and endopeptidases found in blood, tissues and biological fluids [16,22,27,28] (Fig. 4). In plasma and arterial walls, kinins are metabolized by carboxy-terminal degradation [29]. Kininase I (carboxypeptidase N of plasma or carboxypeptidase M of cell membranes) removes the C-terminal Arg and generates the des-Arg-kinin homologues. Kininase II (also named angiotensin-I-converting enzyme, ACE), a dipeptidyl carboxypeptidase, liberates the C-terminal Phe^8-Arg^9 and Ser^6-Pro^7 in a sequential order [16]. Kininase I metabolites are also kininase II substrates [27].



	ENZYME	TISSUE
Kininase I	Carboxypeptidase N Carboxypeptidase M	Serum [30] cell membrane [31]
Kininase II	Angiotensin converting enzyme (ACE)	Membrane,Serum, Endothelium, Brain, Kidney, Lung, cerebrospinal fluid [32,33]

FIGURE 4. Principal kininases: Their cleaving sites for bradykinin and kallidin, the tissues where high activites are found [16].

1.4.3. BIOLOGICAL EFFECTS

The biological effects of kinins include contraction or relaxation of smooth muscle, effects on epithelial ion transport, actions on endothelial cells and promotion of arteriolar vasodilation. Kinins participate in the acute inflammatory response of microvasculature and aid in tissue repair [34]. The control of cell function and mitogenesis are generally mediated via stimulation of B_2 receptors [35-37].

1.4.4. KININ RECEPTORS

Kinins interact with their cell surface G-protein coupled receptors (Fig. 5) to produce a variety of biological effects, such as vasodilation, stimulation of cell proliferation, production of pain and inflammatory responses [22].

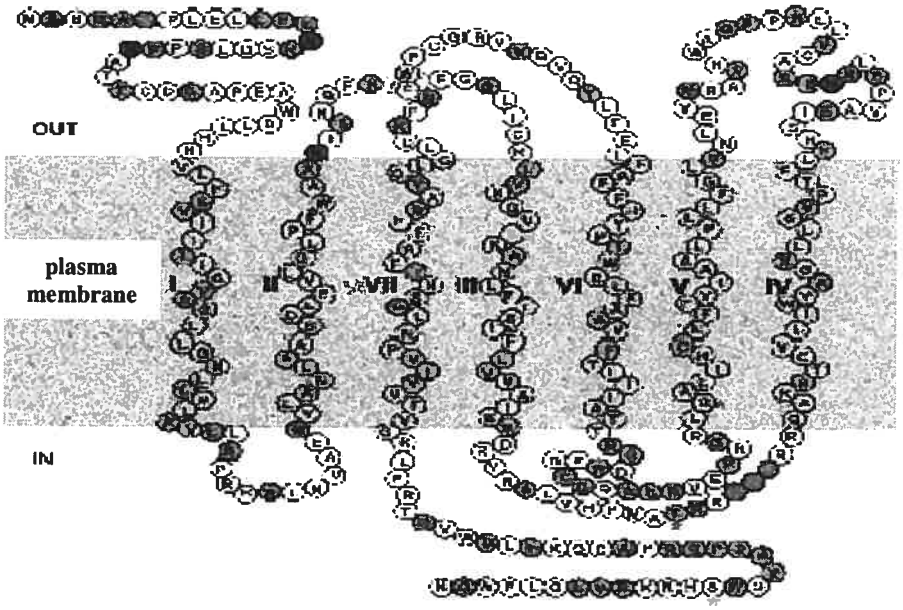


FIGURE 5. The dog B_1 receptor amino acid sequence. Each circle represents an amino acid residue with the single letter code inset. The transmembrane domains are designated by Roman numerals. Clear circles indicate identical residues between dog and human B_1 receptors. Asterisks designate potential phosphorylation sites. Modified from molecular cloning and pharmacological characterization of the canin B1 and B2 bradykinin receptors. Biol. Chem. 382: 123-129 [17].

There are at least two types of kinin receptors. They are designated B_1 and B_2 receptors [18,38]. Although, the existence of a B_3 receptor was suggested in the pulmonary tissue of guinea pig, but later the same group showed that it is invalid [39,40]. By *in situ* hybridization the B_1 receptor gene was localised to human chromosome 14 q 32.1-q32.2 [38], in close proximity to the human B_2 receptor gene mapped to

chromosome14 in band q32 [41,42]. The sequence of kinin receptors has seven hydrophobic segments which form transmembrane helices [34].

B_1 receptors were described for the first time in the isolated rabbit aorta [35]. They have now been documented in isolated preparations taken from the cardiovascular, urinary as well as from intestinal systems, and in cultured cells of vascular, endothelial, mesangial, tracheal, bone and fibroblast origin [35,36,37,43]. Although the B_1 receptor is constitutively present and functional in the canine cardiovascular system [44], in the vas deferens and stomach of the mouse [45], this receptor is generally under-expressed in normal conditions but it is highly inducible by inflammatory mediators such as bacterial lipopolysaccharide, interleukins, overproduction of cytokines and growth factors [20,46].

B_2 receptors have been identified for the first time in rabbit isolated jugular veins using pharmacological *in vitro* assays [35]. B_2 receptors mediate the majority of *in vivo* effects of kinins, including bronchoconstriction, hypotension, acute inflammatory reactions, pain and hyperalgesia [36,47]. Both B_1 as well as B_2 receptors have been shown to mediate vasodilation [34].

A comparison of B_1 and B_2 receptors according to size and interspecies homology is presented in Table IV [16]. The amino acid sequence of the human B_1 receptor (353 amino acid protein) is 36% identical to the amino acid sequence of the human B_2 receptor (364 amino acid protein). The percent homology between human and rat B_2

receptors is 80.6 and B_1 receptors is 70.6. The natural agonists for B_2 receptors are BK and KD, and for B_1 receptors the natural agonists are des-Arg^{10}-KD and des-Arg^{9}-BK. Highly potent and selective peptide as well as non-peptide agonists and antagonists for B_2 receptors and only peptide selective agonists and antagonists for B_1 receptors are available. No nonpeptide B_1 receptor antagonists have been described [7]. The peptide antagonist D-Arg-[Hyp^{3},Thi^{5},D-Tic^{7},Oic^{8},des-Arg^{9}]-BK([desArg^{10}]-Hoe140 has been introduced as a selective B_1 receptor antagonist [48], and Hoe-140 as a selective B_2 receptor antagonist [49].

TABLE IV. BIOCHEMICAL FEATURES AND PHARMACOLOGY OF KININ RECEPTORS [11]

Receptor	B_2	B_1
Family	Rhodopsin superfamily of G-protein-coupled receptors [34]	Rhodopsin superfamily of G-protein-coupled receptors [34]
Number of amino acids (molecular wt.)	rat 366 (41.0 to 41.7 kDa) [50] human 364 (41.0 to 41.5 kDa) [51] mouse 366 (41.5 kDa) [52] rabbit 367 (41.5 kDa) [53]	rat 337 (38.4 kDa) [16] human 353 (40.4 kDa) [54] mouse 334 (38kDa) [55] rabbit 352 (39.5 kDa) [56]
Homology (%)		
Between human and rat	80.6	70.6
Between human and mouse	81.7	72.9
Between human and rabbit	81.7	76.4
Between rabbit and rat	79.8	69.7
Between rabbit and mouse	82.3	74.2
Between mouse and rat	90.7	88.4
Signal transduction mechanism	Phospholipase A_2, C and D, cAMP, cGMP, ion channels	Phospholipase A and C
Order of potency of natural agonists	BK>>des-Arg^9-BK KD>>des-Arg^{10}-KD	des-Arg^9-BK>>BK des-Arg^{10}-KD>>KD
Peptide-selective agonist	Ph$^8\Psi$(CH$_2$NH)Arg^9-BK [57]	Sar[D-Phe^8]des-Arg^9-BK [58]
Non-peptide-selective agonist	FR190997	not available
Peptide-selective antagonist	Hoe-140 [49].	[Leu^8]des-Arg^9-BK [35] Lys [Leu^8]des-Arg^9-BK [35] AcLys[D-βNal^7,Ile^8]des-Arg^9-BK [59]
Non-peptide-selective antagonist	FR 173657 FR 167344 LF 16.0335 Bradyzide	not available

- Hoe-140 is $_D$-Arg-[Hyp^3, Thi^5,$_D$-Tic^7, Oic^8]-BK [49].
- FR190997 is (8-[2,6-dichloro3-[N-[(E)-4(N-methylcarbamoyl)cinnamidoacetyl]-N-methyl-amino]benzyloxy]-2-methyl-4-(2-pyridylmethoxy)quinoline) [60].
- FR167344 is (3-bromo-8-[2,6-dichloro-3-[N-[(E0-4-(n,n-dimethylcarbamoyl)cinnamidoacetyl]-N-methylamino]benzyloxy]-2-methylimidazo[1,2-a]pyridine hydrochloride) [61].
- FR173657 is (8[3-[N[(E)-3-(6-acetamidopyridin-3-yl)acryloylglycyl]-N-methyllamino]-2,6-dichlorobenzyloxy]-2-methylquinoline) [61].
- LF16.0335 is (1-[[3-[(2,4-dimethylquinolin-8-yl)oxymethyl]-2,4-dichloro-phenyl]sulphonyl]-2(S)-[[4-[(aminoiminomethyl)phenylcarbonyl]piperazin-1-yl]carbonyl]pyrrolidine) [62].
- Bradyzide is ((2S)-1-[4-(4-benzhydrylthiosemicarbazido)-3-nitrobenzenesulfonyl]pyrrolidine-2-carboxylic acid[2-(2-dimethyl-aminoethyl)methylamino]ethyl]amide) [63].

1.5. VASCULAR SIGNAL TRANSDUCTION PATHWAYS

The signal initiated by the binding of kinins to their receptors on the cell surface is transduced by G-proteins to intracellular pathways [34] (Fig. 6). In general, kinin receptors activate Gαq/11 and Gαi to stimulate intracellular pathways [16,64].

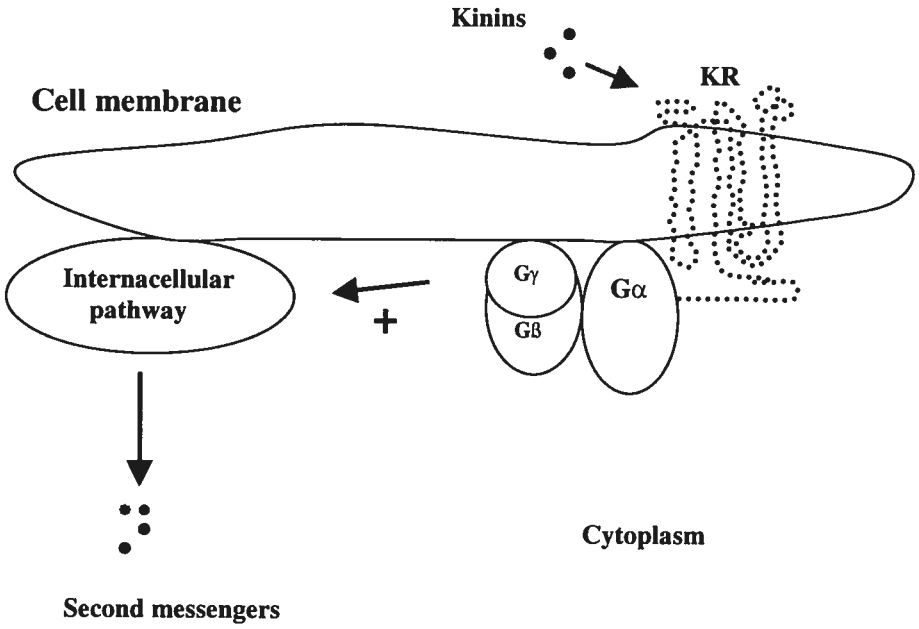


FIGURE 6. The pathway for G-protein dependent signal transduction. KR, kinin receptors; G, G protein incluing its three subunites (a, ß and γ). See text for details [65].

Kinins stimulate a variety of intracellular pathways depending on cellular types. Activation of intracellular pathways by kinins results in the accumulation of cAMP or cGMP, activation of phospholipases A_2, C or D. These second messengers will then produce the release of prostaglandins or NO, the opening of ion channels, and

release of inositol phosphates or DG from membrane inositol phospholipids. The result is mobilization of $[Ca^{2+}]_i$ and activation of several isoforms of PKC [22,66,67]. Moreover, it is also suggested that the activation of protein tyrosine kinases and phosphatases as well as a MAP kinase are involved in kinin-mediated signal transduction [68-70].

1.5.1. KININ-STIMULATED RELEASE OF NITRIC OXIDE

Kinins could stimulate L-arginine through the kinin receptors on the surface of endothelial cells to release NO [22,71]. Nitric oxide is a lipophilic compound, therefore it does not require a cell surface receptor to mediate its action on vascular smooth muscle. Indeed, NO produced by the endothelium probably moves by diffusion into the contiguous vascular smooth muscle. The NO derived from L-arginine activates GC in smooth muscle cells leading to accumulation of cGMP which mediates vascular relaxation [72] (Fig. 7).

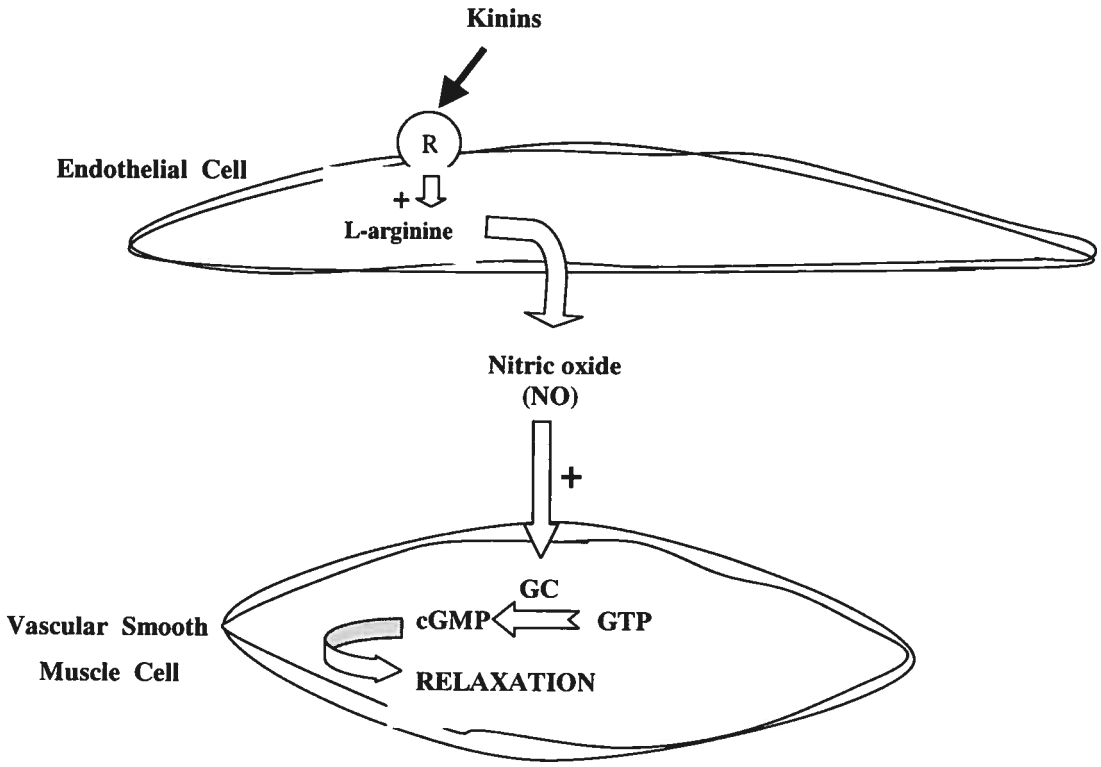


FIGURE 7. Nitric oxide and vascular smooth muscle cells. Production of endothelium-derived nitric oxide from L-arginine. R, kinin receptors ; NO, nitric oxide; GC, guanylyl cyclase; cGMP, cyclic guanosine monophosphate; GTP, guanosine triphosphate [72].

1.5.2. KININ-STIMULATED INCREASE OF INTRACELLULAR CALCIUM

Kinin-stimulated increase of $[Ca^{2+}]_i$ can be achieved via extracellular influx or through release of Ca^{2+} from intracellular stores [73]. There are five possible routes for Ca^{2+} entry into endothelial cells including receptor-operated, voltage-gated Ca^{2+} channels, Ca^{2+} leak channels, stretch-activated Ca^{2+} channels, and Na^+/Ca^{2+} exchange [73]. Kinins increase Ca^{2+} influx into endothelial cells by opening ion channels [73]. Furthermore, mediated by the IP_3 pathway, endothelial cell Ca^{2+} can be increased by kinins (Fig. 8). Following stimulation of kinin receptors, PLC acts upon PIP_2 to generate DG and IP_3. Subsequently, IP_3 mobilizes intracellular Ca^{2+} from internal stores to induce its effects [73]. While a DG-activated PKC allows plasma membrane influx of extracellular Ca^{2+} [73].

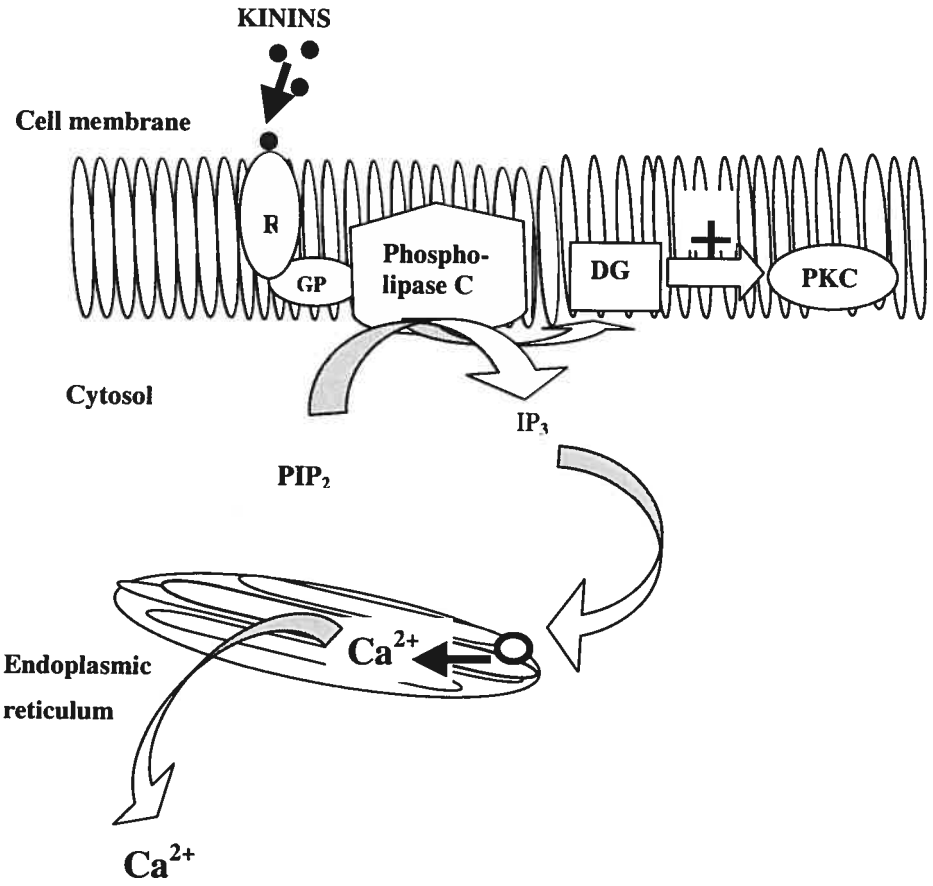


FIGURE 8. The phosphatidylinositol pathway. R, kinin receptors; GP, G-protein; PIP_2, phosphatidyl-inositol 4,5-diphosphate; IP_3, inositol triphosphate; DG, diacylglycerol; PKC, protein kinase C [73].

1.5.3. KININ-STIMULATED RELEASE OF PROSTACYCLIN

Kinins could stimulate phospholipids through kinin receptors on endothelial cells to produce AA, a 20 carbon, unsaturated, fatty acid formed from phospholipids in the cell membrane by PLA_2 . Subsequently, AA is metabolized by cyclo-oxygenases including COX1, a constitutively expressed isoform that is involved in a range of physiological functions, and the COX2 isoform which is inducible and expressed by extracellular stimuli such as pro-inflamatory cytokines in different cells. Further along the pathway, endoperoxides are metabolized by prostacyclin synthetase to produce PGI_2 (Fig. 9a & 9b)[73]. The synthesis of PGI_2 occurs predominantly in the endothelium and causes an increase of AC activity leading to the formation of vasodilator cAMP from ATP [73].

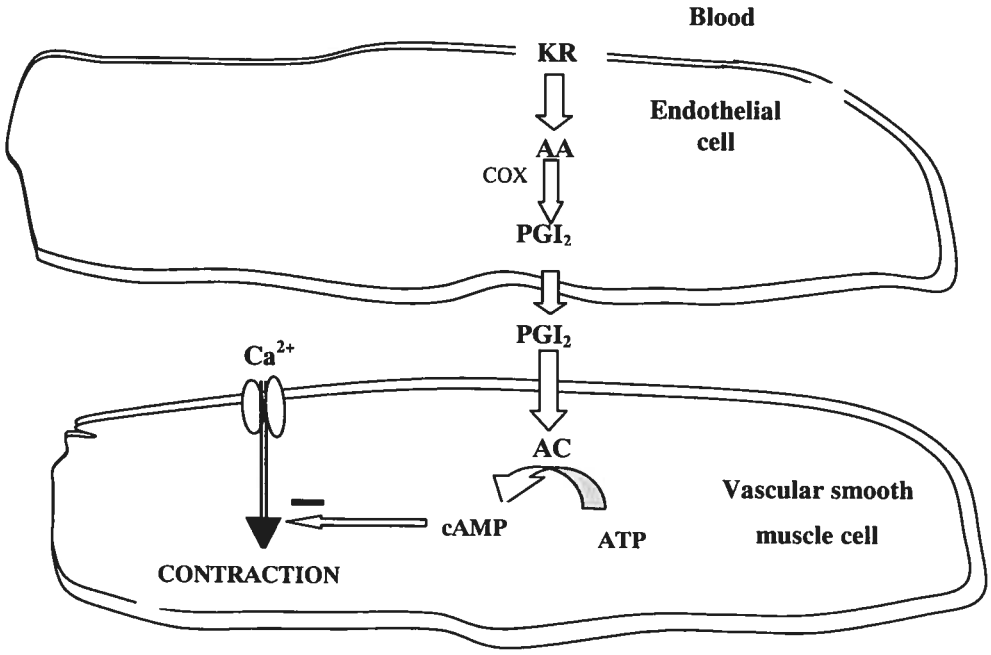


FIGURE 9a. Prostacyclin (PGI_2) and vascular smooth muscle cells. Kinins cause the release of metabolites of Arachidonic acid (AA), PGI_2 , through activation of its specific endothelial receptor. AC, adenylate cyclase; cAMP, cyclic adenosin monophosphate; ATP, adenosine triphosphate; KR, kinin receptors; COX, cyclo-oxygenases [73].

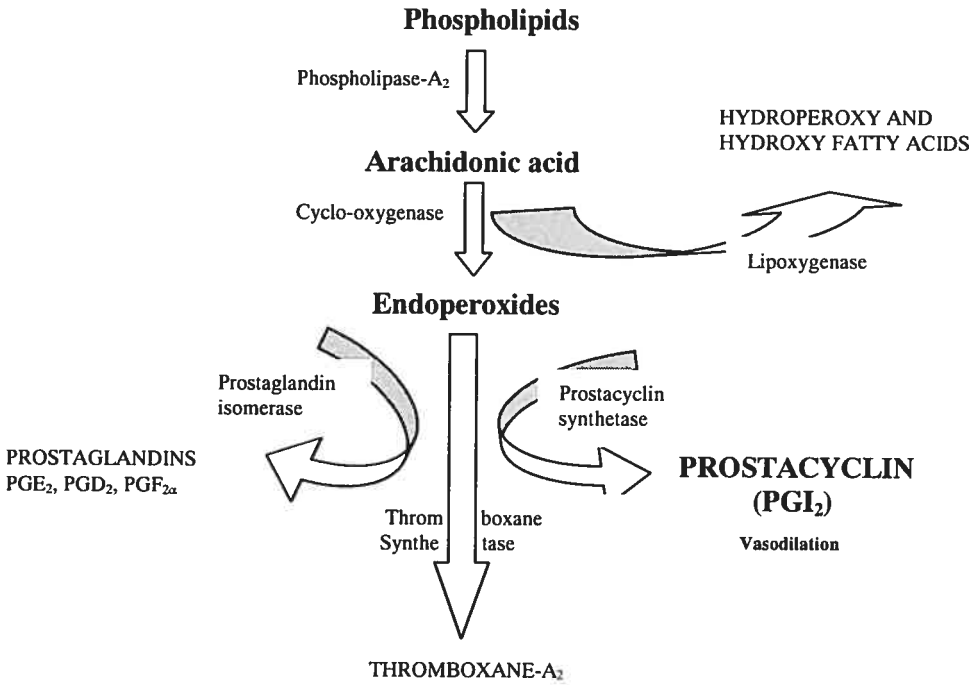


FIGURE 9b. Arachidonic acid and its metabolites [73]. See text for information.

1.6. THE KALLIKREIN-KININ SYSTEM IN RELATION TO THE EYE

Previous studies have demonstrated that the kallikrein-like enzymatic activities exist in tissue homogenates of rabbit and swine eyes [74,75]. Later, another group, using RT-PCR, Southern blot analyses, and *in situ* hybridization histochemistry, identified the expression and localization of components of the kallikrein-kinin system in human retina, choroid, cillary body, optic nerve, and endothelial cells of ocular blood vessels [76]. To date, there has not been a study on the physiological function of the kallikrein-kinin system in ocular tissues and its potential roles in the development of the diabetic retinopathy.

2. HYPOTHESIS

Kinins are present in the retina, and kinins are known to be strong vasodilators. I hereby propose the hypothesis that B_2 receptors mediate vasodilation in the healthy rat, and that early in diabetes kinin B_1 receptors are induced and potentiate the vasodilator effect of kinins in the retinal circulation.

3. AIM

To investigate the capacity for and mechanism of vasodilation produced by kinins in the retinal circulation of diabetic rats and their healthy control.

4. SPECIFIC OBJECTIVES

My specific objectives are to:

1) investigate the effect of BK and des-Arg^9-BK on retinal vessel tone in the healthy and STZ diabetic rat.
2) establish the receptors which mediate the vascular effects of kinins in the healthy and diabetic retina.
3) determine the signal transduction pathways which mediate the vascular effects of kinins in the retinal circulation.

CHAPTER II

1. MATERIALS & METHODS

1.1. BIOLOGICAL MODEL

Male Wistar rats (150-250g); age (37-52 days), were purchased from Charles River (Saint Constant, Québec) and used as the control group. They were kept in our animal facilities at 22 °C, 12 h light/dark cycle and fead with Rodent Laboratory Chow # 5001 (Purina company, Canada) *ad libitum*.

For the diabetic group, Wistar rats were fasted overnight and made diabetic following intraperitoneal injection of a single dose of STZ (65 mg/kg) dissolved in sodium citrate (pH 4.5). STZ is known to cause DNA alkylation and subsequent DNA strand breaks in pancreatic islet cells. This induces an insulitis and produces a model of insulin-dependent diabetes mellitus [77]. Only rats with blood glucose ≥20 mM were considered diabetic. Also, the same rats showed polyuria after thay became hyperglycemic. To measure the blood glucose, the rat's tail was washed with warm water and dried. Then, it was pricked and its blood glucose measured using test strips (Roche company, Québec).

1.2. EXPERIMENTAL PROTOCOL

The blood glucose of rats was evaluated just before the rats were decapitated to remove their eyes. The eyes were enucleated and placed immediately in ice-cold Krebs buffer (pH 7.4) of the following composition in mM: 120 NaCl, 4.5 KCl, 2.5 $CaCl_2$, 1.0 $MgSO_4$, 27 $NaHCO_3$, 1.0 KH_2PO_4, and 10 glucose. A circular incision was made in the posterior segment of the eye near the ora serrata to remove the anterior segment and vitreous body with minimal handling of the retina. The remaining eyecup was fixed with pins to a wax base containing 270 µl of Krebs buffer (pH 7.35-7.45) and maintained at 37°C. The preparations were allowed to stabilize for 15 mins. The outer vessel diameter was recorded with a video camera and responses were quantified by a digital image analyser (Scion image) [78]. A first measure was made and considered as baseline. Afterwards, U-46619 was used to apply a tone to retinal vessels before measuring the effect of kinins. A concentration-response curve (Fig. 10) was made, and 1 µM U-46619 was considered optimal for the rest of the experiments. In a first set of experiments, the vessels were pretreated with U-46619 (1µM), then cumulative concentration-response curves to BK and des-Arg^9-BK in the control and diabetic groups were made. The agonists or antagonists were used and vascular diameter was recorded before and after topical application of each concentration of agent, at which time a stable response was achieved. The responses are expressed as percent change in the outer diameter of the vessel compared to U-46619 constricted vessels. It should be noted that in the present study, our aim was to investigate the changes of diameter of the retinal veins. Veins

have a larger overall diameter than corresponding arteries. Therefore, we chose the vessels which appeard to have larger diameter.

1.3. CHEMICAL AGENTS

For this study, we used the following products: BK, a peptide-selective agonist for the B_2 receptor [57]; des-Arg^9-BK, a peptide-selective agonist for the B_1 receptor [58] ; Hoe-140, a peptide-selective antagonist for the B_2 receptor [49] ; and des-Arg^{10}-Hoe-140, a peptide-selective antagonist for the B_1 receptor [48] were purchased from Peninsula laboratories (California). $GdCl_3$, a channel blocker [79] ; STZ, a DNA alkylating agent [75] ; U-46619, a stable thromboxane A_2 mimetic and a vasoconstrictor agent [80] ; L-NAME, irreversible inhibitor of eNOS and a reversible inhibitor of iNOS[81] ; L-745.337, a specific COX-2 inhibitor [82]; TPC, a PGI_2 synthase inhibitor [83] and BHQ, specifically mobilizes Ca^{2+} from IP_3-sensitive Ca^{2+} stores by inhibiting microsomal and sarcoplasmic reticulum Ca^{2+}-ATPase activity [84] were purchased from Sigma. cADP-ribose which functions as a physiological regulator of Ca^{2+} release and which mobilizes Ca^{2+} from internal pools that are distinct from IP_3–sensitive pools [85] and NF023, a selective and direct G-protein antagonist for α-subunits of the G_o/G_i group [86] were from Calbiochem (California).

1.4. STATISTICAL ANALYSIS

Each set of experiments were conducted on 3-8 rats, where n represents the number of rats which were used. All results are expressed as mean ± SEM. Results are analysed using Student's t-test for paired samples and a one-way analysis of variance (ANOVA) followed by the Dunnett test was used for multiple comparisons with control group. A probability of $p<0.05$ was considered statistically significant.

CHAPTER III

1. RESULTS

The results of this study are divided into two parts. The first part has been conducted in the healthy rat and the second part in the STZ-diabetic rat. Throughout the experiments, U-46619 was used to give vessels tone and thus allow vasodilation to be studied. In an initial set of experiments, a dose-response curve was constructed for U-46619 to set the optimal concentration to use in the experiments. U-46619 was applied serially to retinal preparations with concentrations ranging from 1 nM to 1 mM (Fig. 10). U-46619 induced a dose-dependent vasoconstriction of rat retinal vessels. The vasoconstrictor effect was significant ($p<0.05$, $n=4$) at concentrations ≥100 nM. The optimal concentration of U-46619 was decided to be 1 μM which induces vascular tone similar to phsiological levels.

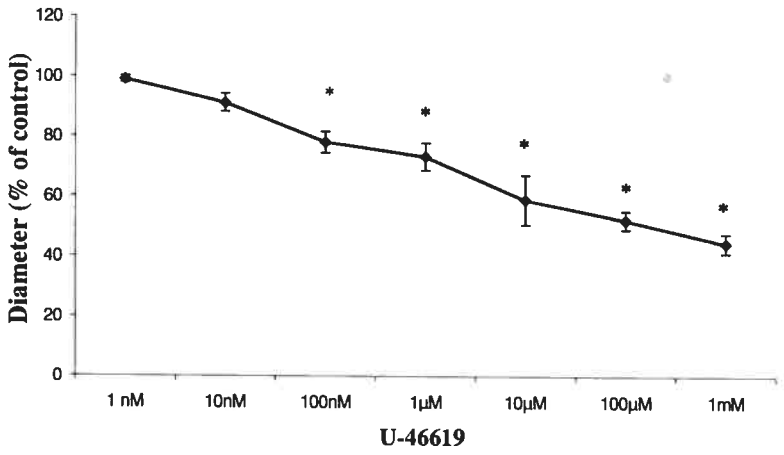


FIGURE 10. Dose-response curve for the effect of U-46619 on retinal vessel diameter. U-46619, was applied at a serial concentration from 1 nM to 1mM for 10 mins to apply tone to the retinal vessels. Results are expressed as mean ± SEM of data obtained from 4 separate retinas (4 rats). (*$p<0.05$, $n=4$) compared to the baseline measure.

1.1. CONTROL RAT

The following experiments were done in control healthy rat retinas, with the aim to investigate the effect of BK on retinal vessel diameter

1.1.1. EFFECT OF BRADYKININ ON RETINAL VESSEL TONE

After a baseline measure was taken, and U-46619 (1μM) was applied, retinas were treated with serial concentrations of BK ranging from 100 pM to 10 nM. Treatment duration for each concentration was 15 mins (Fig. 11). The results show that BK induces a dose-dependent relaxation of retinal vessels following the vasoconstrictor effect of U-46619. The vasodilator effect of BK is significant ($p<0.05$, $n=13$) starting at a concentration of 100 pM.

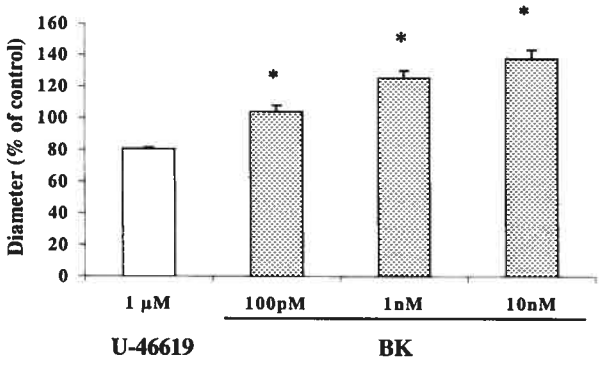


FIGURE 11. Effect of BK on the control rat retinal vessel diameter. A baseline measure was obtained. Then, U-46619 (1μM) was used and the second measure was obtained, after, BK was applied at a serial concentration from 100 pM to 10 nM for 15 mins and the diameter of the vessels was measured each time. (*$p<0.05$, $n=13$) compared to the vasocostrictor effect of U-46619.

1.1.2. EFFECT OF des-Arg^9-BRADYKININ ON RETINAL VESSEL TONE

To determine the effect of des-Arg^9-BK on control rat retinal vessel diameter, U-46619 was used to constrict retinal vessels after which serial concentrations of des-Arg^9-BK were applied over concentrations ranging from 10 pM to 10 nM, with a treatment duration of 15 mins (Fig. 12). Results show that des-Arg^9-BK does not affect ($p>0.05$, $n=7$) retinal vessel diameter following the vasoconstrictor effect of U-46619. Even at a concentration 100 times higher than what is needed of BK to induce retinal vasodilation (des-Arg^9-BK (10 nM) vs BK (100 pM)).

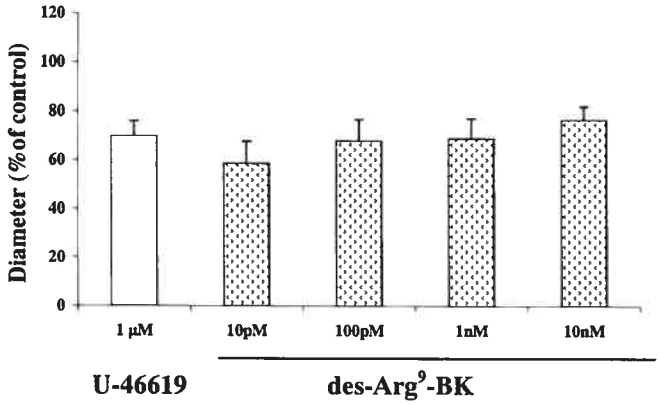


FIGURE 12. Effect of des-Arg^9-BK on the control rats retinal vessel diameter. A baseline measure was obtained and then U46619 was used after 10 mins the second measurement was done, after that a serial concentration of des-Arg^9-BK ranging from 10 pM to 10 nM with a treatment duration of 15 mins was added. The diameter of the vessels was measured each time. The effect of des-Arg^9-BK compared with the vasoconstrictor effect of U-46619 (n=7).

1.1.3. B_2 RECEPTOR ANTAGONISM

To determine which kinin receptor mediates vasodilation evoked by BK under healthy conditions, the following set of experiments were conducted. Retinal vessels were vasoconstricted using U-46619 (1 μM). Afterwards, retinas were pretreated with Hoe-140 (10 μM), the B_2 receptor specific antagonist, for 15 mins, before adding BK (10 nM) (Fig. 13). As the results show, Hoe-140 was without effect on retinal vessel tone. More importantly, BK failed to dilate the vessels pretreated with Hoe-140 ($p<0.01$, $n=7$) when compared to the vasodilator effect of BK alone (Fig. 11). This suggests that B_2 kinin receptors participate in BK-induced retinal vasodilation.

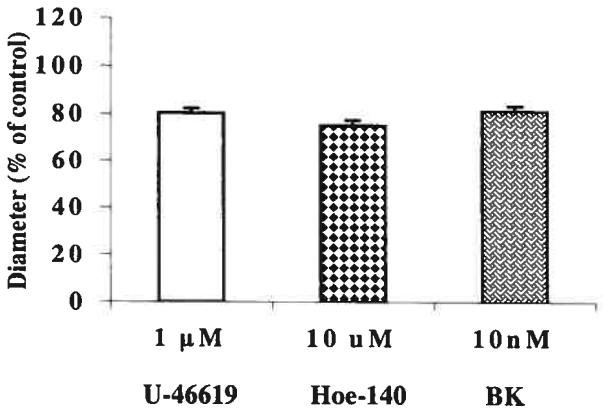


FIGURE 13. B_2 kinin receptors in BK induced control rat retinal vasodilation. A baseline measure was obtained, then U-46619 (1μM) was used after 10 mins the second measurement was done, Hoe-140 (10 uM) was applied and after 15 mins the third measurement was obtained, BK (10 nM) was used and final measurement was done. The effect of BK is suppressed by Hoe-140 (n=7).

1.1.4. B_1 RECEPTOR ANTAGONISM

To address further the question of whether or not B_1 receptors are involved in BK-induced retinal vessel dilation, the following set of experiments were performed. We used the B_1 receptor specific antagonist, des-Arg^{10}-Hoe-140. Retinal vessels were vasoconstricted by U-46619 (1 μM). Then, they were pretreated with des-Arg^{10}-Hoe-140 (10 μM) for 15 mins before adding BK (10 nM) (Fig. 14). The results show that des-Arg^{10}-Hoe-140 was without effect on retinal vessel tone. Nevertheless, in contrast to the effect of Hoe-140, des-Arg^{10}-Hoe-140 failed to inhibit the effect of BK (n=5). This suggests that BK-induced retinal vasodilation is not mediated by the B_1 kinin receptor.

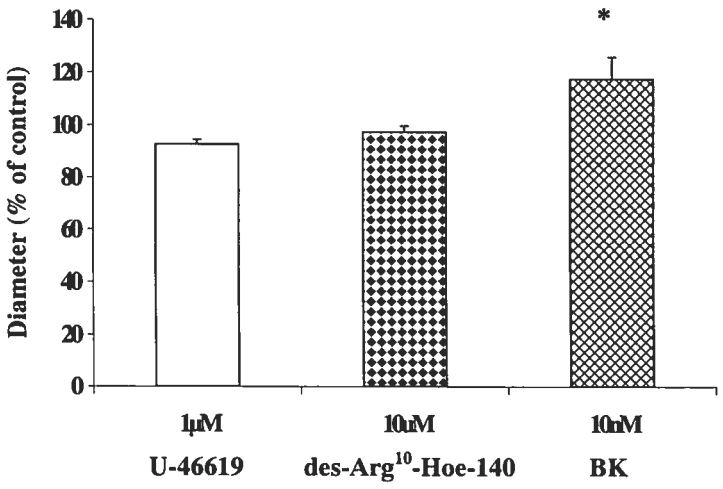


FIGURE 14. B_1 kinin receptors in BK induced control rat retinal vasodilation. A baseline measure was obtained, then U-46619 (1 μM) was used after 10 mins the second measurement was done, des-Arg^{10}-Hoe-140 (10 μM) was applied and after 15 mins the third measurement was obtained, BK (10 nM) was used and final measurement was done. The effect of BK is suppressed by des-Arg^{10}-Hoe-140 (n=5).

1.2. INTRACELLULAR AND MEMBRANE PATHWAYS WHICH MEDIATE THE KININ EVOKED VASODILATION IN CONTROL RATS

In the following series of experiments, the intracellular and membrane pathways which mediate the effect of BK were investigated. To identify these signal transduction pathways we used a battery of signal transducer inhibitors.

1.2.1. G_o/G_i-PROTEINS

Using NF023 we investigated the participation of G_o/G_i-proteins in the vasodilation evoked by BK. Retinal vessels were vasoconstricted with U-46619 (1 μM). Then, they were pretreated with NF023 (100 μM) for 25 mins before adding BK (10 nM) (Fig. 15). The result shows that NF023 was without effect on retinal vessel diameter, and that BK failed to dilate these vessels ($p<0.01$, $n=4$) when compared to the vasodilator effect of BK in the absence of NF023 (Fig. 11). This demonstrates that the vasodilator effect of BK is transduced by G_o/G_i-proteins.

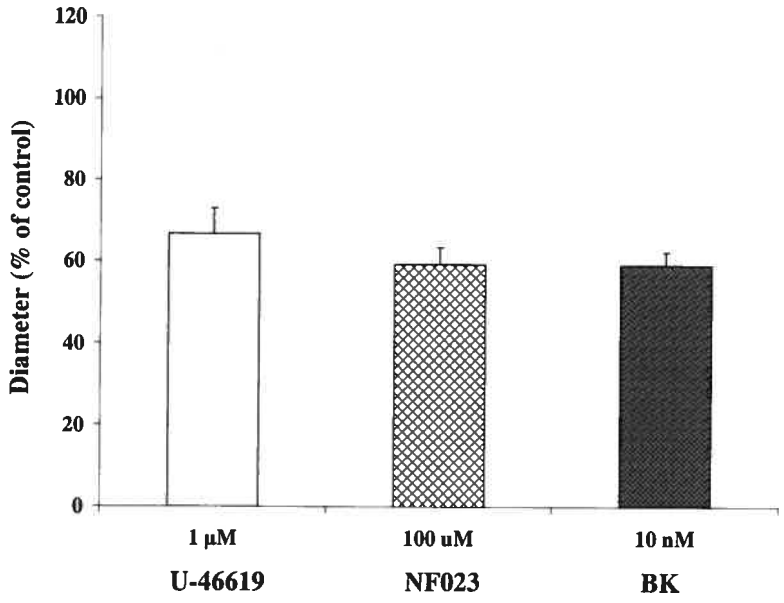


FIGURE 15. G_o/G_i-proteins in BK induced control rat retinal vasodilation. A baseline measure was obtained, then U-46619 (1µM) was used after 10 mins the second measurement was done, NF023 (100 µM) was applied and after 25 mins the third measurement was obtained, BK (10 nM) was used and final measurement was done. The effect of BK is suppressed by NF023 (n=4).

1.2.2. NITRIC OXIDE SYNTHASE INHIBITION

To determine whether or not NO mediates the BK evoked vasodilation, L-NAME was used to inhibit NOS. Retinas were exposed to U-46619 to apply vascular tone. Afterwards, the preparation was pretreated with L-NAME (100 μM), for 20 mins before adding BK (1 nM) (Fig.16). The results obtained from these experiments show that L-NAME was without effect on retinal vessel diameter. However, BK induced a significant vasodilation ($p<0.05$, $n=5$) in the retina. Therefore, NO is not involved in the vasodilator effect of BK in the retina.

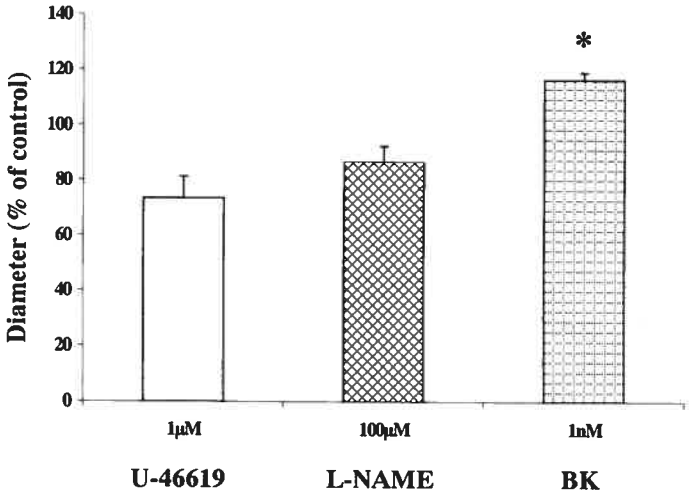


FIGURE 16. Nitric oxide in BK induced control rat retinal vasodilation. A baseline measure was obtained. Then, U-46619 (1μM) was used after 10 mins the second measurement was done. After that , L-NAME (100 μM) was applied for 20 mins, then the third measurement was obtained. Finally, BK (1 nM) was used and the final measurement was done. The vasodilator effect of BK is not blocked by L-NAME. (*$p<0.05$, $n=5$).

1.2.3. CALCIUM CHANNEL BLOCKERS

Is Ca^{2+} influx necessary for the BK evoked vasodilation? To address this question, $GdCl_3$ was used to block the Ca^{2+} influx pathway of endothelial cells. Retinas were exposed to U-46619 to provide a tone to vessels. Then, they were treated with $GdCl_3$ (10 mM) for 15 mins before addition of BK (1 nM) (Fig. 17). The results show that $GdCl_3$ was without effect on retinal vessel tone compared to the vasoconstrictor effect of U-46619. However, BK induced a significant vasodilation ($p<0.05$, $n=5$). This suggests that Ca^{2+} influx is not required for BK to induce vasodilation in the retina.

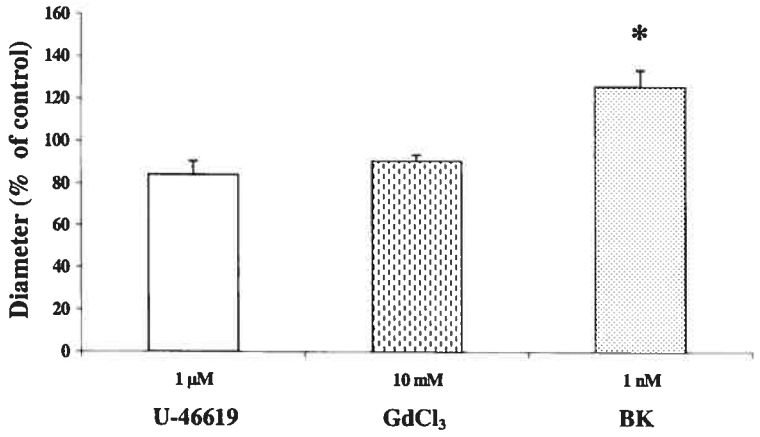


FIGURE 17. Extracellular calcium influx in BK induced control rat retinal vasodilation. A baseline measure was obtained. Then, U-46619 (1 µM) was used after 10 mins the second measurement was done. Afterwards, $GdCl_3$ (10 mM) was applied for 15 mins, then the third measurement was obtained. Finally, BK(1 nM) was used and the final measurement was done. The $GdCl_3$ did not block the effect of BK. (*$p<0.05$, $n=5$).

1.2.4. IP_3-SENSITIVE INTRACELLULAR CALCIUM STORES

To verify whether IP_3-sensitive Ca^{+2} stores are involved in the effect of BK, we used BHQ. As BHQ was dissolved in ethanol, experiments were done to verify if the same concentration of ethanol which was used as vehicle affects the retinal vessel response to BK. Retinal vessels were vasoconstricted using U-46619 (1 μM). Afterwards, retinas were pretreated with ethanol (0.0001%) for 10 mins before adding BK (1 nM) (Fig. 18). When compared to the vasoconstrictor effect of U-46619 the results show that ethanol is not able to affect the diameter of these vessels, and BK induces a significant vasodilation ($p<0.05$, $n=5$) in the presence of ethanol. This shows that the ethanol vehicle does not affect retinal vessel responses to BK. After these control studies, BHQ was tested on the retina (Fig. 19). Following treatment with U-46619 (1 μM), retinas were pretreated with BHQ (1 μM) for 10 mins before the addition of BK (1 nM). As the results show, BHQ does not change the diameter of vessels when compared to U-46619. Moreover, the vasodilator effect of BK was inhibited by BHQ ($p<0.01$, $n=6$) (Fig. 11), which suggests that the release of Ca^{+2} from IP_3-sensitive intracellular Ca^{+2} stores is implicated in the vasodilator effect of BK.

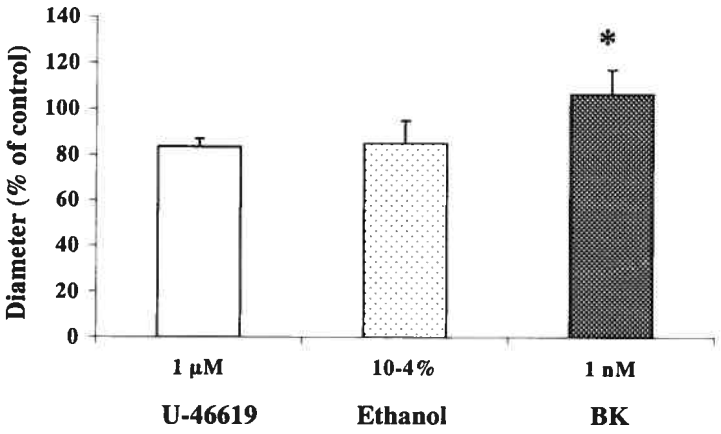


FIGURE 18. Ethanol (vehicle for BHQ) in BK induced control rat retinal vasodilation. A baseline measure was obtained. Then, U-46619 (1 μM) was used after 10 mins the second measurement was done. Afterwards , ethanol (10^{-4}%) was applied for 10 mins, then the third measurement was obtained. Finally, BK (1 nM) was used and the final measurement was done, (*$p<0.05$, n=5).

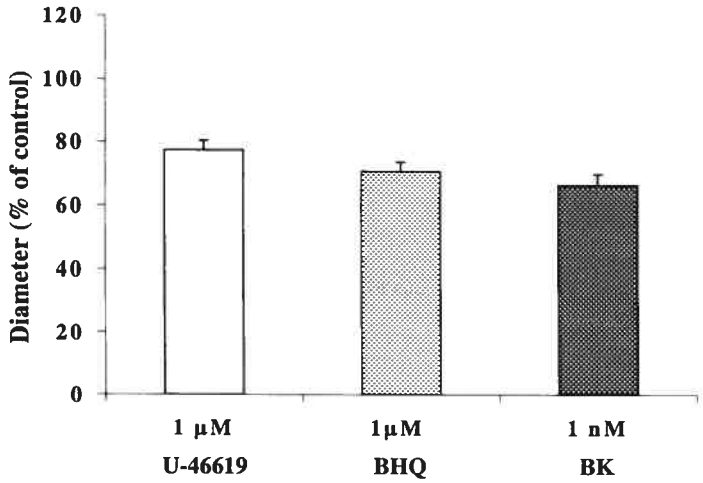


FIGURE 19. IP_3-sensitive intracellular calcium stores in BK induced control rat retinal vasodilation. A baseline measure was obtained. Then, U-46619 (1 μM) was used after 10 mins the second measurement was done. Afterwards , BHQ (1 μM), was applied for 10 mins, then the third measurement was obtained. Finally, BK (1 nM) was used and the final measurement was done (n=6).

1.2.5. NON-IP_3-SENSITIVE INTRACELLULAR CALCIUM STORES

In the following set of experiments, cADP-ribose was used to investigate the role of IP_3-insensitive intracellular Ca^{2+} pool in BK-induced rat retinal vasodilation. Retinal vessels were vasoconstricted by U-46619 (1 μM). Then, the vessels were treated with cADP-ribose (10 μ M) for 25 mins before addition of BK (10nM) (Fig.20). The results show that cADP-ribose does not affect the diameter of the vessels compared to the vasoconstrictor effect of U-46619. Morever, the vasodilator effect of BK is blocked ($p<0.01$, n=5) when compared to the vasodilator effect of BK alone (Fig. 11). This suggest that IP_3-insensitive intracellular Ca^{2+} pools are implicated in the effect of BK.

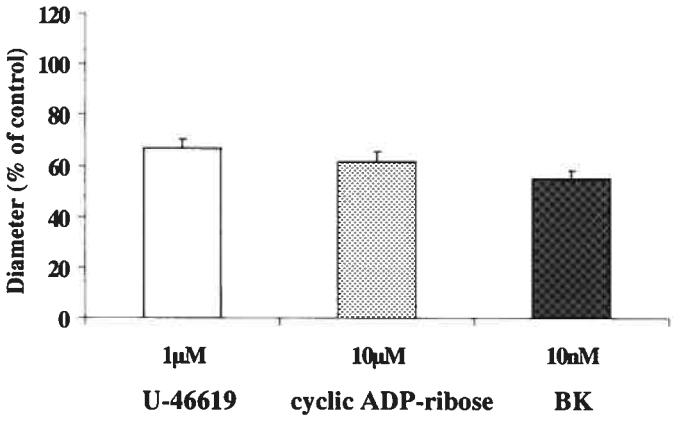


FIGURE 20. Non-IP_3-sensitive intracellular calcium stores in BK induced control rat retinal vasodilation. A baseline measure was obtained. Then, U-46619 (1 μM) was used after 10 mins the second measurement was done. Afterwards , cyclic ADP-ribose (10 μM), was applied for 25 mins, then the third measurement was obtained. Finally, BK(10 nM) was used and the final measurement was done (n=5).

1.2.6. COX-2 INHIBITORS

To determine whether or not products of the cyclooxygenase pathway are involved in the retinal vasodilator effect of BK, we used L-745.337, a specific COX-2 inhibitor. As L-745.337 is soluble in DMSO, control experiments were done to verify that a same concentration of DMSO as was used for a vehicle does not affect retinal vessel tone. Retinal vessels were vasoconstricted by U-46619 (1 μM). Then, they were treated with DMSO (0.0015%) for 30 mins before addition of BK (1 nM) (Fig. 21). The results show that DMSO does not affect vessel diameter, and that BK can induce vasodilation ($p<0.05$, $n=3$). In the second set of experiments, retinal vessels were vasoconstricted using U-46619 (1 μM). Then, they were pretreated with L-745.337 (1 μM) for 30 mins before adding BK (1 nM) (Fig.22). The results show that L-745.337 does not affect the diameter of vessels. Moreover, BK can not induce retinal vessel dilation ($p<0.01$, $n=8$) when compared to the vasodilator effect of BK in the absence of L-745.337 (Fig. 11). This suggests that the cyclooxygenase pathway is involved in the retinal vasodilator effect of BK.

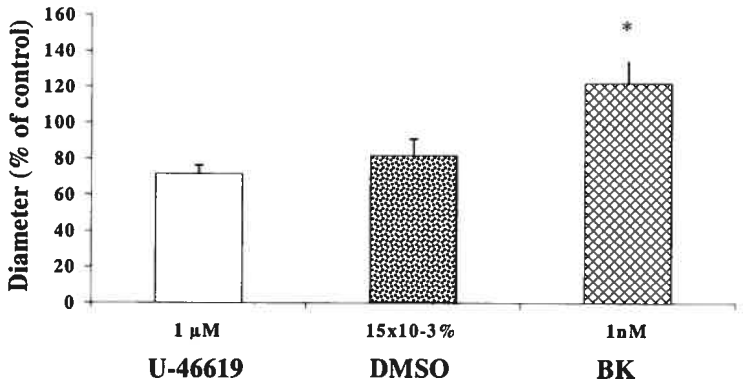


FIGURE 21. DMSO (vehichle for L-745.337) in BK induced control rat retinal vasodilation. A baseline measure was obtained. Then, U-46619 (1 µM) was used after 10 mins the second measurement was done. Afterwards, DMSO (15x 10^{-3} %) for 30 mins was applied, then the third measurement was obtained. Finally, BK (1 nM) was used and the final measurement was done. The effect of BK is present. (*p<0.05, n=3).

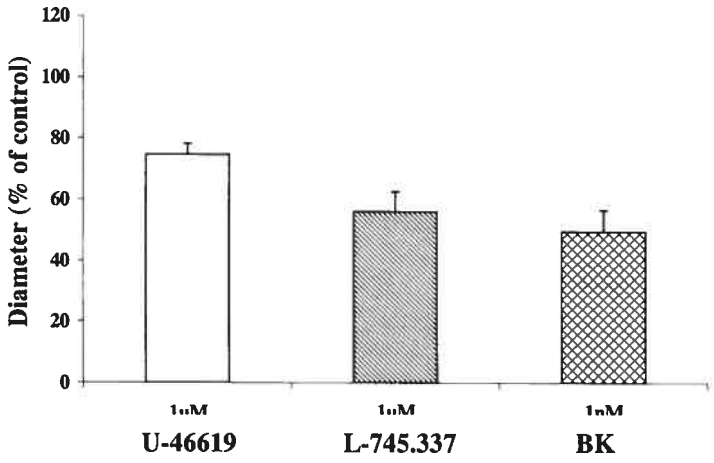


FIGURE 22. The COX-2 pathway in BK induced control rat retinal vasodilation. A baseline measure was obtained. Then, U-46619 (1µM) was used after 10 mins the second measurement was done. Afterwards, L-745.337 for 30 mins was applied, then the third measurement was obtained. Finally, BK (1 nM) was used and the final measurement was done (n=8).

1.2.7. PROSTACYCLIN SYNTHASE INHIBITORS

To verify whether or not PGI_2 is involved in the retinal vasodilator effect of BK we used TPC. Retinal vessels were vasoconstricted with U-46619 (1 μM). Then, they were pretreated with TPC (5 μM) for 25 mins before adding BK (1 nM) (Fig. 23). The results show that TPC did not affect the diameter of vessels. Morever, BK was not able to dilate retinal vessels ($p<0.01$, $n=6$) when compared to the vasodilator effect of BK in the absence of TPC (Fig. 11). This suggests that PGI_2 is implicated in the vasodilator effect of BK in the retina.

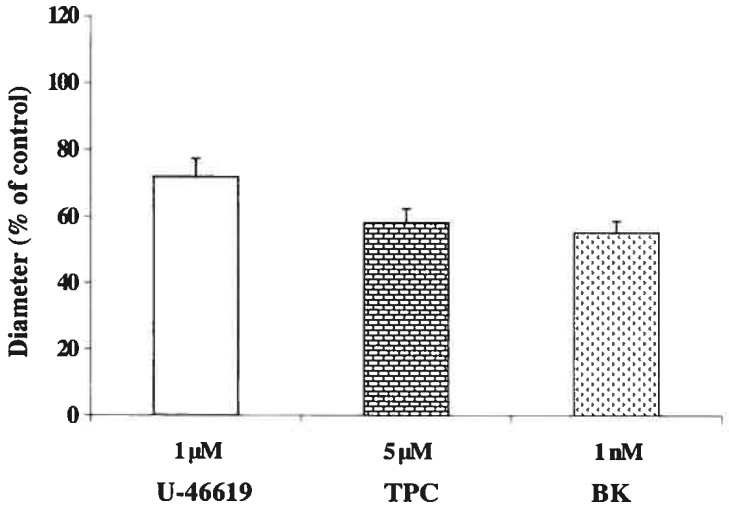


FIGURE 23. Prostacyclin in BK induced control rat retinal vasodilation. A baseline measure was obtained. Then, U-46619 (1μM) was used after 10 mins the second measurement was done. Afterwards, TPC (5 μM) for 25 mins was applied, then the third measurement was obtained. Finally, BK (1 nM) was used and the final measurement was done (n=6).

In summary, our data from the control rat group show that:

i) BK dilates retinal vessels and Hoe-140 inhibits the vasodilation induced by BK,

ii) des-Arg^9-BK is without effect on retinal vessel diameter,

iii) the vasodilator effect of BK is mediated by stimulation of B_2 receptors, but not B_1 receptors,

iv) the signal initiated via stimulation of B_2 receptors by BK is transduced by G_o/G_i-proteins,

v) NO and extracellular Ca^{2+} influx are not involved in the effect of BK,

vi) the products of the cyclooxygenase pathway are involved in the retinal vasodilator effect of BK,

vii) PGI_2 mediates the vasodilation, and

viii) intracellular Ca^{2+} pools from both IP_3-sensitive and IP_3 insensitive stores are necessary for the vasodilator effect of BK.

1.3. STREPTOZOTOCIN-DIABETIC RAT

In this section, we will show the kinin effects on retinal vessel diameter in STZ-diabetic rats. Rats were made diabetic as described in section II.1.1. Rats injected with STZ but which did not develop diabetes (blood glucose ranging from 5 to 10 mM) were taken as controls to verify any potential direct cytotoxic effect of STZ on the retina. It should be stressed that those rats which became diabetic (blood glucose >20 mM), did so as early as 1 day after injection of STZ.

1.3.1. EFFECT OF des-Arg^9-BRADYKININ ON RETINAL VESSEL TONE

To investigate what is the effect of des-Arg^9-BK on STZ-diabetic rat retinal vessel tone, rats were put into 6 separate groups. The first group received normal saline (control group), the second group were STZ-injected rats which did not become diabetic, and the other four groups are STZ-injected diabetic rats: 1 day, 4, 7 and 21 days after the administration of STZ. After each period of treatment, the eyes have been prepared as was previously described. Retinas were exposed to U-46619 (1μM) to apply a tone to the vessels. Ten minutes later, des-Arg^9-BK was used in serial concentrations ranging from 10 pM to 1 nM. The duration of treatment with des-Arg^9-BK was for 15 mins (Fig.24). In the control group which received normal saline (n=7) and in the STZ-injected non-diabetic rat group (n=5), des-Arg^9-BK did not affect the diameter of vessels when compared to the vasoconstrictor effect of U-46619. Also, in 1 day STZ-injected diabetic rats, des-Arg^9-BK did not affect the diameter of the vessels ($p>0.05$, n=3) when compared to the vasoconstrictor effect of U-46619. However, in the other groups, 4 days (n=6), 7 days (n=6), and 21 days (n=5), des-Arg^9-BK changed the diameter of the vessels significantly ($p<0.05$) and dose-dependently when compared to the vasoconstrictor effect of U-46619.

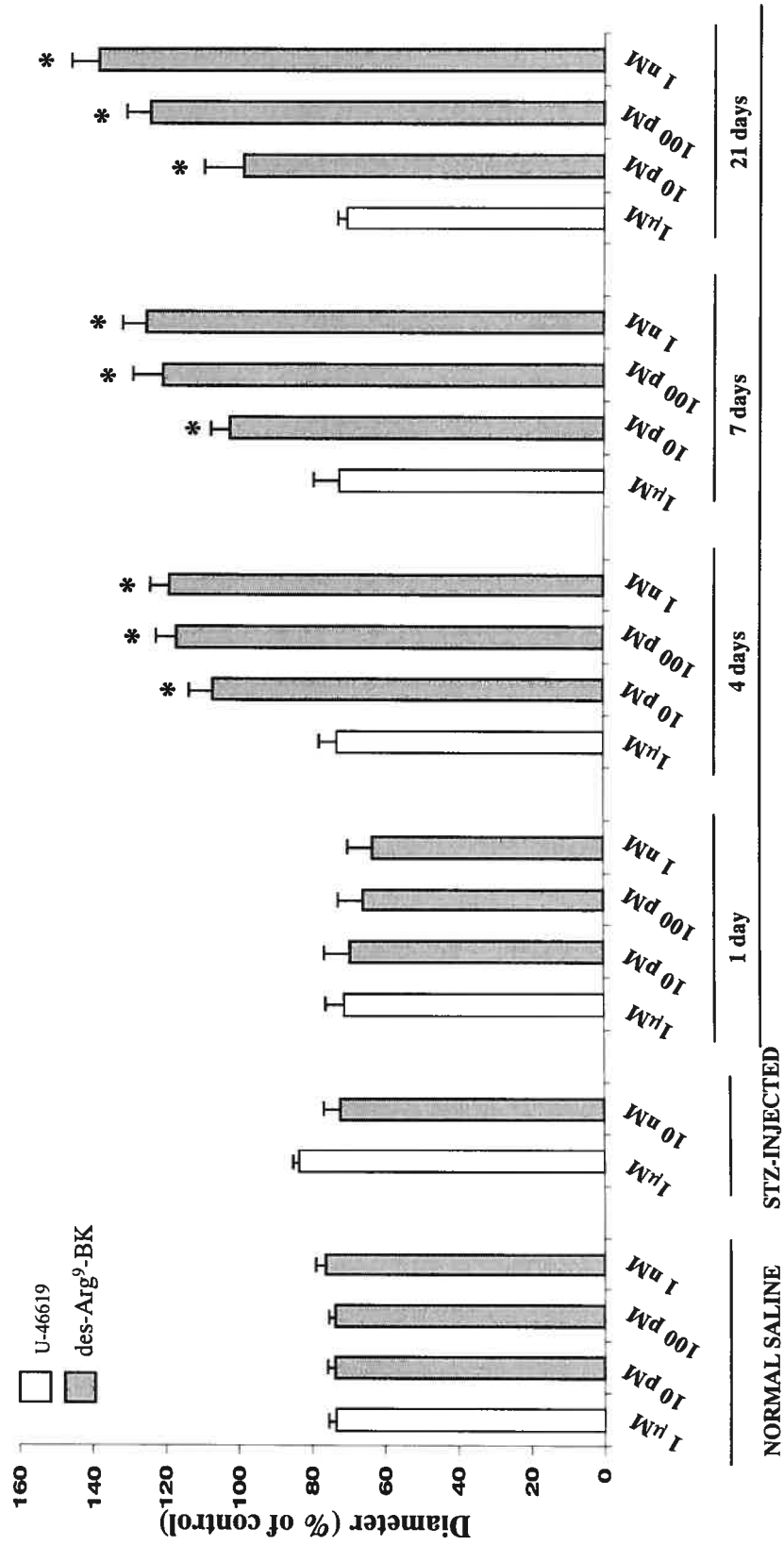


FIGURE 24. Effect of des-Arg^9-BK on STZ-diabetic rat retinal vessel diameter. The results from 6 separate groups: The control group received normal saline (n=7), the second group contains rats which received STZ without developing diabetes (n=5), and the other groups which received STZ and became diabetic. In this order: 1 day (n=3); 4 days (n=6); 7 days (n=6) and 21 (n=5) after injection.were used. In each group, a baseline measure was obtained. Then, U-46619 (1µM) was used and the second measure was obtained, after that , des-Arg^9-BK was applied at a serial concentration from 10 pM to 1 nM for 15 mins and the diameter of the vessels was measured each time.*$p<0.05$ compared to the vasocostrictor effect of U-46619.

1.3.2. EFFECT OF BRADYKININ ON RETINAL VESSEL TONE

The effect of BK on retinal vessel tone in STZ-diabetic rats was verified as follows. STZ-diabetic rat retinal vessels were vasoconstricted with U-46619 (1μM) for 10 mins. Then, BK (10 nM) was added. (Fig 25). The results show that BK induces relaxation in the retinal vessel when compared with the vasoconstrictor effect of U-46619 ($P<0.05$, n=3). This result suggests that BK is able to change the diameter of the vessels in STZ-diabetic rat.

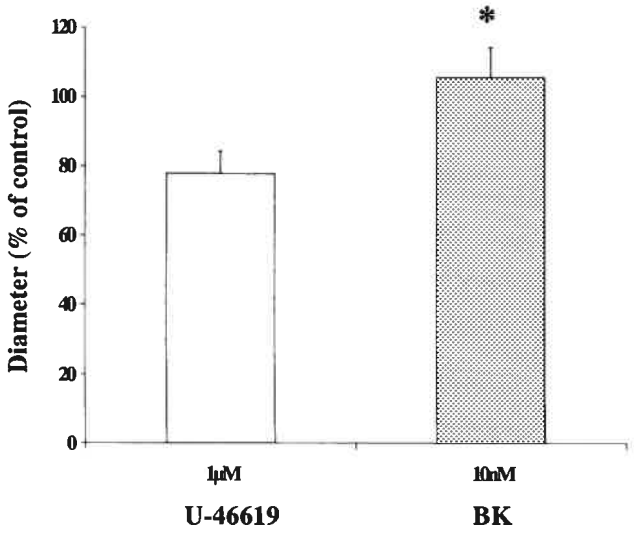


FIGURE 25. Effect of BK on STZ-diabetic rat retinal vessel diameter. A baseline measure was obtained. Then, u-46619 (1μM) was used and the second measure was obtained, after that, BK (10nM) for 15 mins and the diameter of the vessels was measured (*$P<0.05$, n=3).

1.3.3. RESPONSE TO des-Arg^9-BRADYKININ FOLLOWING B_1 RECEPTOR ANTAGONISM

For the purpose of identifying which receptor is stimulated by des-Arg^9-BK on 21 day-STZ-diabetic rat retinal vessels to induce vasodilation, the B_1 receptor specific antagonist, des-Arg^{10}-Hoe-140, was used. The vessels were exposed to U-46619 (1 μM) for 10 mins to apply tone. Then, they were pretreated with des-Arg^{10}-Hoe-140 (1 μM) for 15 mins before adding des-Arg^9-BK (1 nM) (Fig. 26). The results show that des-Arg^9-Hoe-140 does not affect the diameter of the vessels compared to the effect of U-46619. Moreover, des-Arg^{10}-Hoe-140 blocks the effect of des-Arg^9-BK ($p<0.01$, n=3), which demonstrates that the vasodilator effect of des-Arg^9-BK in retinal vessel is mediated by B_1 receptors.

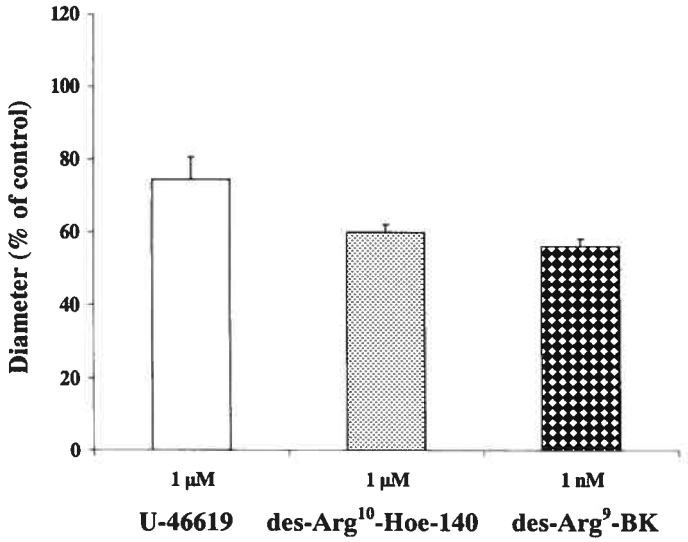


FIGURE 26. B_1 kinin receptors in des-Arg^9-BK induced 21 day-STZ-diabetic rat retinal vasodilation. A baseline measure was obtained. Then U-46619 (1μM) was used after 10 mins the second measurement was done, (des-Arg^{10}-Hoe-140) (1 μM) was applied after 15 mins the third measurement was obtained , des-Arg^9-BK (1 nM) was used and final measurement was done (n=3).

1.3.4. RESPONSE TO BRADYKININ FOLLOWING B_2 RECEPTOR ANTAGONISM

To verify which receptor is stimulated by BK in STZ-diabetic rat retinal vessels to induce vasodilation, we used the specific B_2 receptor antagonist, Hoe-140. The vessels were vasoconstricted by U-46619 (1μM) for 10 mins. Then, the vessels were treated with Hoe-140 (10 μM) for 15 mins before adding BK (10 nM). As the data show, Hoe-140 does not affect the vessels compared to the effect of U-46619. Moreover, Hoe-140 inhibits the effect of BK ($p<0.01$, n=4) (Fig 27). This suggests that the effect of BK is mediated by B_2 receptors on retinal vessels of STZ-diabetic rats.

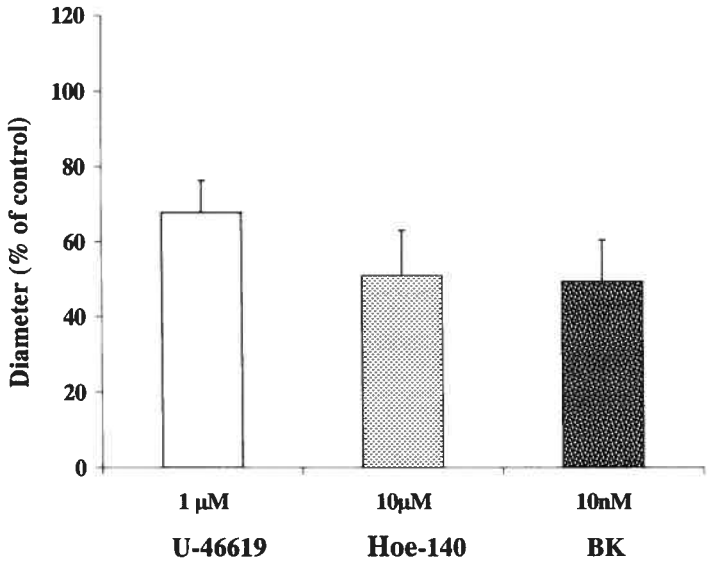


FIGURE 27. B_2 kinin receptors in BK induced STZ-diabetic rat retinal vasodilation. A baseline measure was obtained. Then U-46619 (1μM) was used after 10 mins the second measurement was done, Hoe-140 (10 μM) was applied after 15 mins the third measurement was obtained, BK (10 nM) was used and final measurement was done (n=4).

1.4. INTRACELLULAR AND MEMBRANE PATHWAYS WHICH MEDIATE THE KININ EVOKED VASODILATION IN STZ-DIABETIC RATS

In the following series of experiments, we investigated the intracellular signals which mediate the effect of des-Arg^9-BK. To determine which signal transduction pathways participate in this response we used a battery of inhibitors.

1.4.1. RESPONSE TO des-Arg^9-BRADYKININ FOLLOWING INHIBITION OF G_o/G_i-PROTEINS

To investigate whether or not G_o/G_i-proteins are involved in the des-Arg^9-BK-induced retinal vasodilation we used NF023. Retinal vessels were vasoconstricted using U-46619 (1 μM). Then, they were treated with NF023 (100 μM) for 25 mins before adding des-Arg^9-BK (1 nM) (Fig. 28). The result shows that NF023 was without effect on retinal vessel diameter when compared to the effect of U-46619, and des-Arg^9-BK failed to dilate the vessels ($p<0.01$, $n=3$) when compared to the effect of des-Arg^9-Bk in the absence of NF023 (Fig.24). This demonstrates that des-Arg^9-BK-induced retinal vasodilation is mediated by G_o/G_i-proteins.

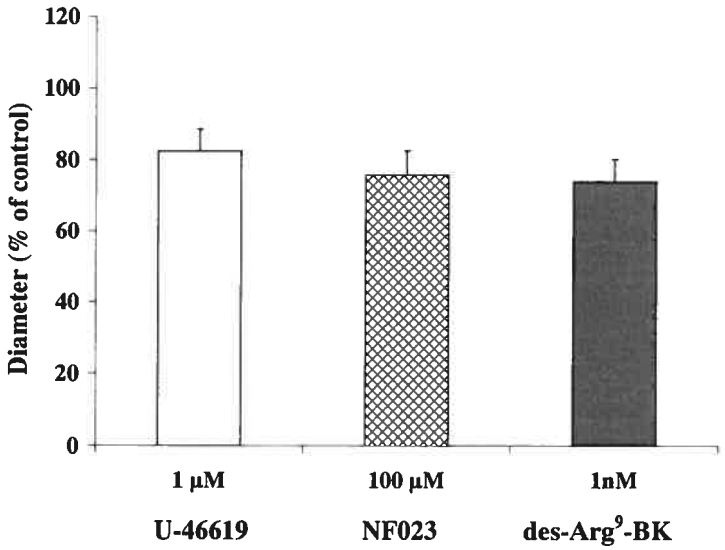


FIGURE 28. G_o/G_i-proteins in des-Arg^9-BK induced STZ-diabetic rat retinal vasodilation. A baseline measure was obtained, then U-46619 (1μM) was used after 10 mins the second measurement was done, NF023 (100 μM) was applied and after 25 mins the third measurement was obtained, des-Arg^9-BK (10 nM) was used and final measurement was done. The effect of des-Arg^9-BK is suppressed by NF023 (n=3).

1.4.2. RESPONSE TO des-Arg^9-BRADYKININ FOLLOWING NITRIC OXIDE SYNTHASE INHIBITION

Does the response to des-Arg^9-BK involve the release of NO in the retinal circulation of the STZ-diabetic rat? To address this question, we used L-NAME. Retinas were exposed to U-46619 to apply tone to the vessels. Afterwards, they were treated with L-NAME (100 μM) for 20 mins before adding des-Arg^9-BK (1 nM) (Fig.29). The results obtained from these experiments show that L-NAME was without effect on retinal vessel diameter. Moreover, L-NAME failed to inhibit des-Arg^9-BK-induced retinal vessel dilation ($p<0.05$, n=3). These results suggest that this pathway is not involved in the vasodilator effect of des-Arg^9-BK in the retina.

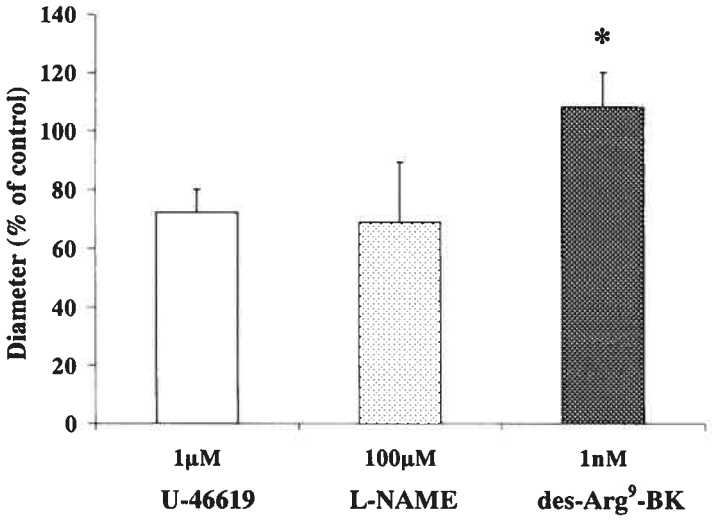


FIGURE 29. Nitric oxide in des-Arg^9-BK induced 7 day-STZ-diabetic rat retinal vasodilation. A baseline measure was obtained. Then, U-46619 (1μM) was used after 10 mins the second measurement was done. After, L-NAME (100 μM) was applied for 20 mins, then the third measurement was obtained. Finally, des-Arg^9-BK (1 nM) was added and the final measurement was done ($p<0.05$, n=3).

1.4.3. RESPONSE TO des-Arg^9-BRADYKININ FOLLOWING CALCIUM CHANNEL BLOCKADE

Does des-Arg^9-BK induce retinal vasodilation by opening Ca^{2+} channels? With the aim to address this question we used $GdCl_3$. Retinas were exposed to U-46619 to apply tone to vessels. Subsequently the vessels were treated with $GdCl_3$ (10 mM) for 15 mins before the addition of des-Arg^9-BK (1 nM) (Fig. 30). The results show that $GdCl_3$ was without effect on retinal vessel diameter when compared to the vasoconstrictor effect of U-46619. Moreover, $GdCl_3$ failed to inhibit des-Arg^9-BK-induced retinal vessel dilation ($p<0.05$, n=6). This suggests that Ca^{2+} influx is not involved in the vasodilator response to des-Arg^9-BK.

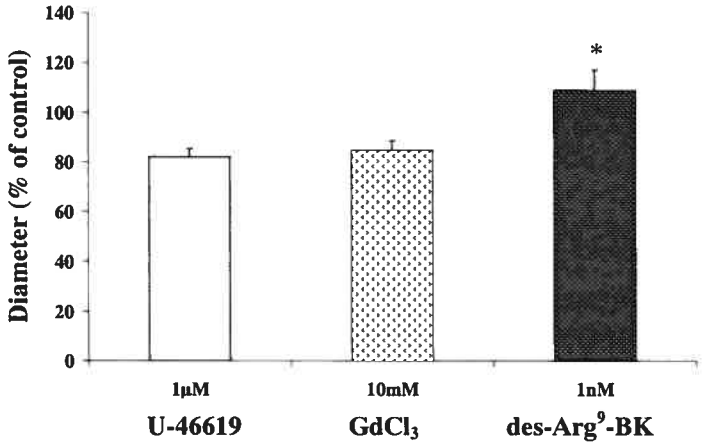


FIGURE 30. Extracellular calcium influx in des-Arg^9-BK induced STZ-diabetic rat retinal vasodilation. A baseline measure was obtained. Then, U-46619 (1μM) was used after 10 mins the second measurement was done. Afterwards, $GdCl_3$ (10 mM) was applied for 15 mins, then the third measurement was obtained. Finally, des-Arg^9-BK (1 nM) was used and the final measurement was done (n=6).

1.4.4. RESPONSE TO des-Arg^9-BRADYKININ FOLLOWING ANTAGONISM OF IP_3-SENSITIVE INTRACELLULAR CALCIUM STORES

Does des-Arg^9-BK stimulate the IP_3 pathway to release Ca^{2+} from the endoplasmic reticulum to affect retinal vessels? To verify whether IP_3-sensitive Ca^{2+} stores are involved in the effect of BK, we used BHQ. As BHQ is soluble in ethanol, control experiments were done to verify if concentrations of ethanol equal to what was used as vehicle affects the retinal vessel response to des-Arg^9-BK. Retinal vessels were vasoconstricted with U-46619 (1 μM). Afterwards, retinas were pretreated with ethanol (0.0001%) for 10 mins prior to the addition of BK (1 nM) (Fig 31). The results show that this concentration of ethanol is not able to affect the diameter of the vessels when compared to the vasoconstrictor effect of U-46619. Furthermore, des-Arg^9-BK induced a significant vasodilation ($p<0.05$, n=4). This shows that ethanol does not affect the retinal vessel response to des-Arg^9-BK. Once this was established, BHQ was tested in the retinal circulation. After treatment with U-46619 (1μM), retinas were pretreated with BHQ (1 μM) for 10 mins before des-Arg^9-BK (1 nM) was added. As the results show, BHQ did not change the diameter of the vessels when compared to U-46619. Moreover, des-Arg^9-BK dose not change the diameter of the vessels ($p<0.01$, n=3) compared to the effect of des-Arg^9-BK in the absence of BHQ (Fig.24). This suggests that the release of Ca^{2+} from IP_3-sensitive intracellular Ca^{2+} stores is implicated in the vasodilator effect of des-Arg^9-BK (Fig.32).

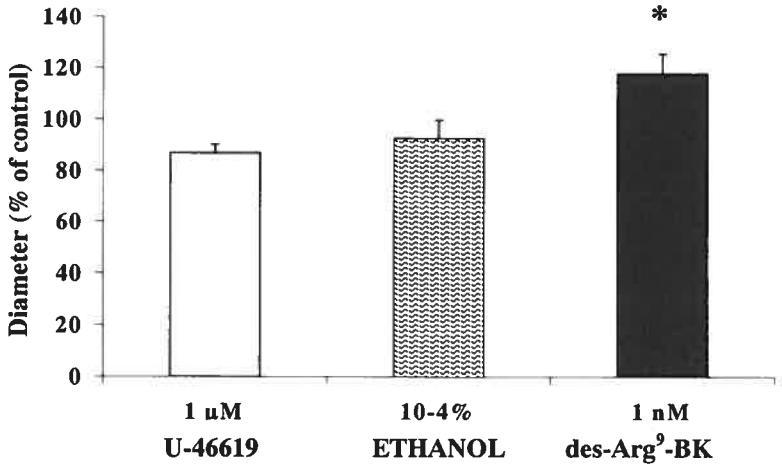


FIGURE 31. Ethanol (vehicle for BHQ) in des-Arg^9-BK induced STZ-diabetic rat retinal vasodilation. A baseline measure was obtained. Then, U-46619 (1µM) was used after 10 mins the second measurement was done. Afterwards, ethanol (10^{-4}%) was applied for 10 mins, then the third measurement was obtained. Finally, des-Arg^9-BK (1 nM) was used and the final measurement was done. ($p<0.05$, n=4).

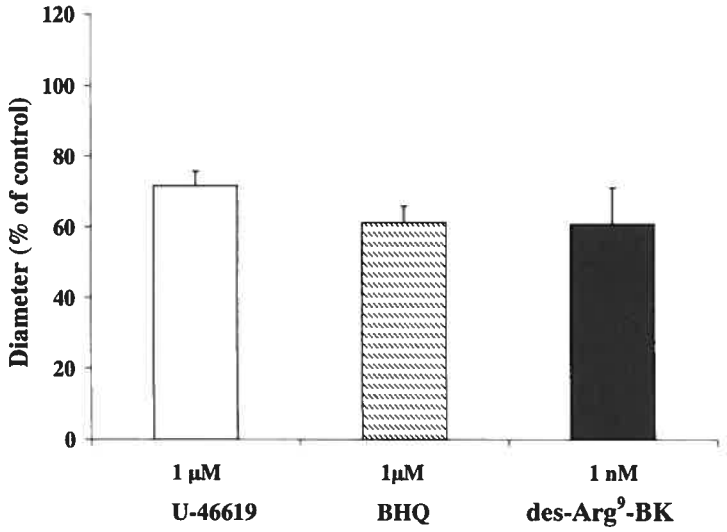


FIGURE 32. IP_3-sensitive intracellular calcium stores in des-Arg^9-BK induced STZ-diabetic rat retinal vasodilation. A baseline measure was obtained. Then, U-46619 (1µM) was used after 10 mins the second measurement was done. Afterwards , BHQ (1 µM), was applied for 10 mins, then the third measurement was obtained. Finally, des-Arg^9-BK (1 nM) was used and the final measurement was done (n=3).

1.4.5. RESPONSE TO des-Arg^9-BRADYKININ FOLLOWING ANTAGONISM OF NON-IP$_3$-SENSITIVE INTRACELLULAR CALCIUM STORES

Do IP$_3$-insensitive intracellular Ca^{2+} pools participate in the effect of des-Arg^9-BK? In the following set of experiments, cADP-ribose was used to investigate the role of IP$_3$-insensitive intracellular Ca^{2+} pools in des-Arg^9-BK-induced rat retinal vasodilation. Retinal vessels were vasoconstricted using U-46619 (1 μM). Then, the vessels were treated with cADP-ribose (10 μM) for 25 mins before addition of des-Arg^9-BK (1nM) (Fig. 33). The results show that cADP-ribose does not affect the diameter of vessels when compared to the vasoconstrictor effect of U-46619. Moreover, the effect of des-Arg^9-BK was blocked ($p<0.01$, n=4) when compared to the effect of des-Arg^9-BK in the absence of cADP-ribose (Fig. 24). This suggests that IP$_3$-insensitive intracellular Ca^{2+} pools are implicated in the effect of des-Arg^9-BK.

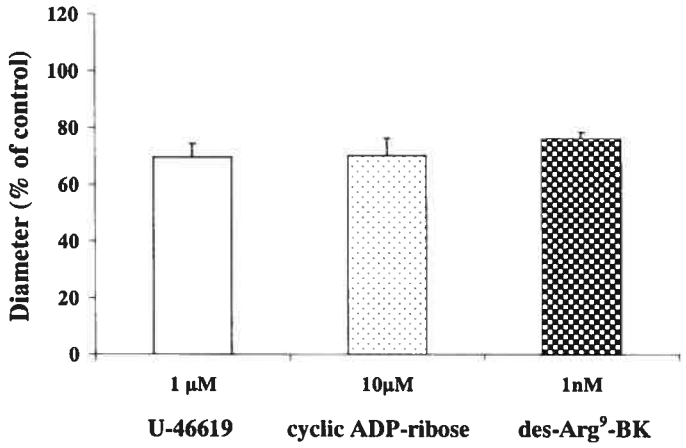


FIGURE 33. Non-IP$_3$-sensitive intracellular calcium stores in des-Arg^9-BK induced STZ-diabetic rat retinal vasodilation. A baseline measure was obtained. Then, U-46619 (1 μM) was used after 10 mins the second measurement was done. Afterwards, cyclic ADP-ribose (10 μM) was applied for 25 mins, then the third measurement was obtained. Finally, des-Arg^9-BK (1 nM) was used and the final measurement was done (n=4).

1.4.6. RESPONSE TO des-Arg^9-BRADYKININ FOLLOWING COX-2 INHIBITION

Are the products of the cyclooxygenase pathway involved in the retinal vasodilator effect of des-Arg^9-BK? For this purpose we used, L-745.337, a specific COX-2 inhibitor. The retinal vessels were vasoconstricted using U-46619 (1 μM). Then, they were treated with L-745.337 (1 μM) for 30 mins before addition of des-Arg^9-BK (1 nM) (Fig 34). The results show that L-745.337 does not affect the diameter of these vessels. Moreover, the effect of des-Arg^9-BK was inhibited ($p<0.01$, n=4) when compared to the effect of des-Arg^9-BK alone (Fig.24). This demonstrates that the cyclooxygenase pathway is involved in the retinal vasodilator effect of des-Arg^9-BK.

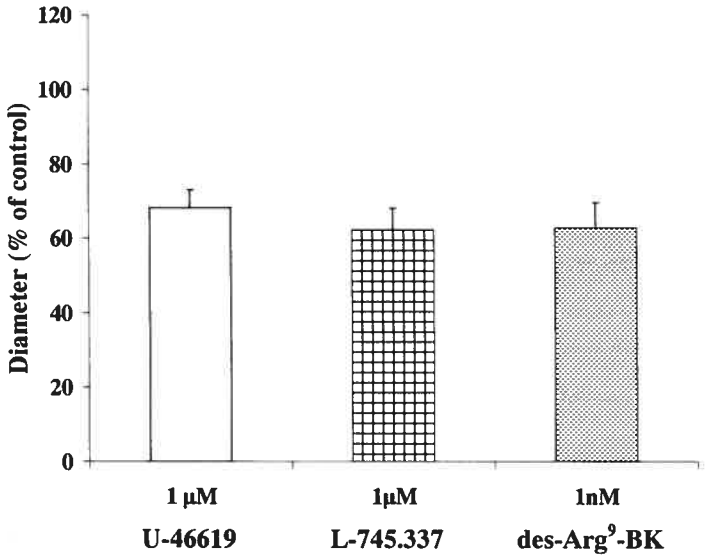


FIGURE 34. The COX-2 pathway in des-Arg^9-BK induced STZ-diabetic rat retinal vasodilation. A baseline measure was obtained. Then, U-46619 (1 μM) was used after 10 mins the second measurement was done. Afterwards, L-745.337 (1 μM) for 30 mins was applied, then the third measurement was obtained. Finally, des-Arg^9-BK (1 nM) was used and the final measurement was done (n=4).

1.4.7. RESPONSE TO des-Arg^9-BRADYKININ FOLLOWING PROSTACYCLIN SYNTHASE INHIBITION

Is PGI_2 involved in the retinal vasodilator effect of des-Arg^9-BK? For this purpose, we used TPC. Retinal vessels were vasoconstricted using U-46619 (1 μM). Then, they were treated with TPC (5 μM) for 25 mins before addition of des-Arg^9-BK (1 nM) (Fig. 35). The results show that TPC did not affect the diameter of these vessels. Moreover, des-Arg^9-BK was not able to dilate retinal vessels ($p<0.01$, n=6) compared to the effect of des-Arg^9-BK in the absence of TPC (Fig. 24). This suggests that PGI_2 is implicated in the vasodilator effect of des-Arg^9-BK in the retina.

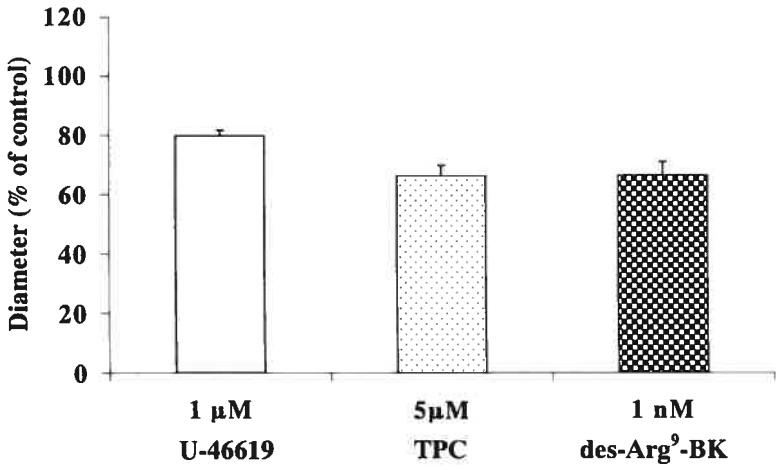


FIGURE 35. Prostacyclin in des-Arg^9-BK induced STZ-diabetic rat retinal vasodilation. A baseline measure was obtained. Then, U-46619 (1μM) was used after 10 mins the second measurement was done. Afterwards, TPC (5 μM) for 25 mins was applied, then the third measurement was obtained. Finally, des-Arg^9-BK (1 nM) was used and the final measurement was done (n=6).

In summary, our data from the STZ-diabetic rat group show that:

i) des-Arg^9-BK induced vasodilation and this effect is mediated by B_1 receptors,

ii) BK dilates retinal vessels and the vasodilator effect of BK is mediated by B_2 receptors,

iii) the signal initiated by activation of B_1 receptors by des-Arg^9-BK is transduced by G_o/G_i-proteins,

iv) NO and extracellular Ca^{2+} influx are not involved in the effect of des-Arg^9-BK,

v) the products of the cyclooxygenase pathway are involved in the retinal vasodilator effect of des-Arg^9-BK,

vi) PGI_2 mediates the vasodilation, and

vii) intracellular Ca^{2+} pools from both IP_3-sensitive and IP_3-insensitive stores are necessary for the vasodilator effect of des-Arg^9-BK.

CHAPTER IV

DISCUSSION

In the present study, we investigated the function of kinins (BK and des-Arg^9-BK) in the retinal circulation of healthy and of STZ-diabetic rats. Furthermore, using a pharmacological approach we investigated the mechanisms which mediate the kinins evoked vasodilation.

Previous studies have shown that the retina, choroid, ciliary body, iris and aqueous humor of swine eyes contain enzymes capable of producing and inactivating kinins (kallikrein and kininase I and II, respectively) [74]. It was demonstrated that the activity of these enzymes varies in different eye structures, the highly vascularized tissues of the retina and choroid have the highest actvities [74]. Furthermore, the activity of tissue as well as plasma kallikrein were studied in rabbit eye structures, and it was found that activity of tissue kallikrein was higher than plasma kallikrein activity in iris, ciliary body, vascular striatum, retina and conjunctiva [75]. Moreover, molecular studies using RT-PCR and Southern blot analysis of the key components of the kallikrein-kinin system including tissue kallikrein, on human ocular tissues, have detected LMWK, and kinin B_1 and B_2 receptors at high levels in human retina, choroid and cillary body, and relatively low levels in the optic nerve [76]. Also, *in situ* hybridization has identified cellular localization of these four mRNAs in ocular tissues. Such mRNA were also identified in endothelial cells of ocular blood vessels, ciliary muscle and lens epithelial cells [76]. These data demonstrate that the kallikrein-

kinin system is present in ocular tissues. A true understanding of the meaning of these findings depends on knowing what is the function of this system in ocular tissues. Furthermore, it is necessary to understand the function of this system under physiological conditions and whether or not this system is involved in retinal vascular complications associated with pathological conditions such as diabetic retinopathy. Indeed, there was no study which addresses the role of the kinins in the regulation of ocular circulation.

Our study is the first to investigate the potential role of kinins in the regulation of retinal vessel tone. The results from the experiments in the control group showed that BK produces significant retinal vasodilation. The ability of such low concentrations (100 pM) of BK to change the diameter of retinal vessels suggests that it can function to regulate retinal vessel tone. BK induced retinal vessel relaxation with an ED_{50} of approximately 250 nM which is in the same range of the K_d for B_2 receptors found in other tissues [34] . A regulatory role of kinins on vessel tone has been demonstrated in other studies. There have been reports of elevated blood pressure in animals or humans genetically deficient in kallikrein, in animals treated acutely or chronically with selective B_2 receptors antagonists, and in genetically engineered animals with a disrupted B_2 receptor gene [87-89]. It was demonstrated that loss of the B_2 receptor in genetically engineered mice causes a slight but significant rise in systemic blood pressure [90]. Other studies demonstrated that ACE inhibition increases kinin levels in tissues and/or plasma [91]. Also, there is a longstanding belief in the literature that at least a part of the beneficial effects of ANG I-converting enzyme

(ACE) inhibitors is derived from the potentiation of endogenous BK. These inhibitors block one of the major inactivation pathways for kinins and lead the kallikrein-kinin system to produce active metabolites instead of inactive metabolites[92]. This pharmacology has been widely used in therapy for hypertension, congestive heart failure, and myocardial infarction [91,92].

Furthermore, our study suggests that BK affects vessel tone in diabetic rats. Altogether, BK not only plays the regulatory role of the kallikrein-kinin system in physiological states but also it could affect the vessels in pathological states. Consequently, the effect of BK may synchronize with the effect of the des-Arg^9-BK in pathological conditions and in this case, BK would not play a role to re-establish balance but it would produce a massive vasodilation early in the evolution of diabetic retinopathy.

When we used 10 pM to 10 nM des-Arg^9-BK in control rats, vessel diameter was not affected. It is known that des-Arg^9-BK has a subnanomolar affinity for B_1 receptors[34]. With this experiment we can suggest that the receptor B_1 which is the specific receptor for des-Arg^9-BK is not expressed under normal conditions. A full verification of this possibility requires receptor studies based on molecular biology and radioligand-binding assays.

In the diabetic group, des-Arg^9-BK induced a significant vasodilation at a concentration of 10 pM. The vasodilation appeared with a delay of about 4 days in

the STZ-diabetic rat. It is in accordance with other studies which demonstrated that in inflamed rat knee joints, following antigen-induced chronic arthritis, there was a delay of about 1 to 5 days in plasma exudation due to kinin stimulated B_1 receptors[92]. In addition, it was found in a rat model of neuropathic hypersensitivity following periphreal nerve injury, that the expression of B_1 receptor mRNA was increased at 14 days post-injury in the site of nerve injury while expression of mRNA for B_2 receptors was already present at 48 h and was expressed bilaterally at 14 days [94].

In our study, we also showed that BK dilates retinal vessels by activating B_2 receptors in control and STZ-diabetic rats. In contrast, des-Arg^9-BK dilated the retinal vessels via the activation of B_1 receptors only in STZ-diabetic rats. These are in accordance with the literature which demonstrates that while B_2 receptors are constantly expressed by several tissues, B_1 receptors are generally not expressed under normal physiological conditions but are rapidly induced during pathological situations such as diabetes [95]. The reports indicate that diabetes can upregulate B_1 receptors through a PKC-activated NF-κB following an autoimmunity response associated with overproduction of cytokines including IL-1ß and TNFα [96], and by oxidative stress following hyperglycemia [5]. Furthermore, the literature shows that STZ, used to produce a model of insulin-dependent diabetes mellitus, causes DNA alkylation and subsequent DNA strand breaks in pancreatic islet cells and induces an insulitis associated with cytokines which can upregulate B_1 receptors [97]. By using RT-PCR analyses and *in situ* hybridization, it was demonstrated that endothelial cells of retinal blood vessels express mRNA for both B1 and B2 receptors [76]. However, there

was no investigation on the translation of the receptor protein or its insertion into the cell membrane. It is possible that even if the B_1 receptor mRNA is transcribed, it would be partially translated or not translated at all because of its instability or an uncoupling with intracellular transducers.

It is documented that kinin receptors activate G-proteins, Gαq/11 and Gαi, to stimulate the intracellular pathways following interaction with their agonists (BK, des-Arg^9-BK) [16,64]. Our results show that the vasodilatory effect of either B_1 or B_2 receptors are transduced by a G_o/G_i-protein. More specific antagonists are needed to make clear which classes are activated by kinin receptors in the retinal vessels.

Various signal transduction pathways have been described for kinins depending on the cell type [22,67-70]. Our results from control and from STZ-diabetic rats show that the release of NO and Ca^{2+} influx are not activated by BK or des-Arg^9-BK. While, the release of intracellular Ca^{2+} from both IP_3-sensitive and IP_3-insensitive intracellular Ca^{2+} pools, and the release of PGI_2 are stimulated by BK and des-Arg^9-BK to induce vasodilation in the retina. This is in accordance with previously reported data showing that the cardiovascular effect of BK dependant vasodilator responses depend on the release of AA from membrane phospholipids and increase of the $[Ca^{2+}]_i$ following stimulation of B_2 receptors by BK [98]. In other studies it was shown, using isolated dog blood vessels, that the vasodilation induced by BK is mediated by the release of PGI_2 from endothelial cells [99]. Furthermore, COX-2 which is inducible following a variety of stimuli including cytokines and immune complexes implicated

in the pathogenesis of type I diabetes mellitus and upregulation of B_1 receptors [96,100,101]. It suggests that both BK and des-Arg^9-BK initiate a complex signalling cascade that causes the release of AA in retinal vessels and consequently the release of PGI_2. This release of AA is dependent on the activation of $cPLA_2$ mediated by a G_o/G_i-protein. This enzyme is thought to be activated following stimulation by agonists such as kinins, that also cause elevations of $[Ca^{2+}]_i$ [65]. BK and des-Arg^9-BK increase the $[Ca^{2+}]_i$ through its release from intracellular sources.

The present study shows the ability of kinins to induce retinal vessel dilation, a process that takes place in the early stages of diabetic retinopathy. Changes in vascular flow and caliber begin very early after weakening of blood vessels due to pericyte degeneration in the early stages of diabetic retinopathy. Pericyte degeneration leads to the overproduction of cytokines and activation of NF-κB which stimulates the kallikrein-kinin system and promotes vasodilation. Furthermore, data from other investigations show that endothelial cell migration and proliferation both *in vivo* and *in vitro* is enhanced by kinins, and that they may promote the angiogenic cascade, with the eventual formation of new capillaries [95]. It was previously concluded that the tissue kallikrein and kinins have a role in the sequential steps that form the angiogenic cascade leading to the formation of new blood vessels [102]. Evidence for a role of B_1 in reparative angiogenesis comes from the observation that the B_1 gene expression is up-regulated by ischemia in skeletal muscle or myocardium [103,104]. It was demonstrated that the endogenous B_1 signaling is essential for developing new blood vessels with functional relevance for recovery

from ischemia[105]. There is a substantial body of evidence supporting a role for kinins in the host defense response to ischemic injury [105]. All of these changes induced by the kallikrein-kinin system in other tissues are the same as changes that may take place during the development of diabetic retinopathy including neovascularization in response to severe ischemia and hypoxia of the retina. These findings reveal the need for more investigation on the kallikrein-kinin system, and in particular its implication in the later stages of diabetic retinopathy.

Our results suggest that the kallikrein-kinin system plays a crucial role in the regulation of blood flow in the retina and this system could have a role in the early stages of the development of diabetic retinopathy. The kinin system could be the basis for a prophylactic approach to prevent the later complications of diabetes mellitus in the retina. The action of kinins could be inhibited by the use of antagonists or inhibitors to block the intracellular pathways which are stimulated by kinin receptors or by the use of enzymes such as kininase II or angiotensin-I-converting enzyme, ACE.

CONCLUSION

This is the first study which shows an effect of kinins in the retinal circulation. The present study shows that : BK dilates retinal vessels in control and STZ-diabetic rats. The vasodilator effect of BK is mediated by stimulation of B_2 receptors. des-Arg^9-BK is without effect on retinal vessel diameter in control healthy rats, but it dilates retinal vessels of STZ-diabetic rats and its effect is mediated by stimulation of B_1 receptors. Neither the release of NO nor Ca^{2+} influx are not involved. In contrast, the release of PGI_2 from AA and the increase of intracellular Ca^{2+} derived from both IP_3-sensitive and IP_3-insensitive intracellular Ca^{2+} pools are implicated in the vasodilator effect of BK and des-Arg^9-BK.

These data suggest a function for kinins in the retinal circulation (Fig.36). In healthy rats, BK stimulates B_2 receptors, whereas in STZ-diabetic rats des-Arg^9-BK stimulates B_1 receptors and BK stimulates B_2 receptors. Throughout, the signals are transduced by G-proteins (G_o/G_i) and the release of PGI_2 from the AA. This release is dependent on the activation of $cPLA_2$. In addition, BK elevates intracellular Ca^{2+} from both IP_3-sensitive and IP_3-insensitive Ca^{2+} stores.

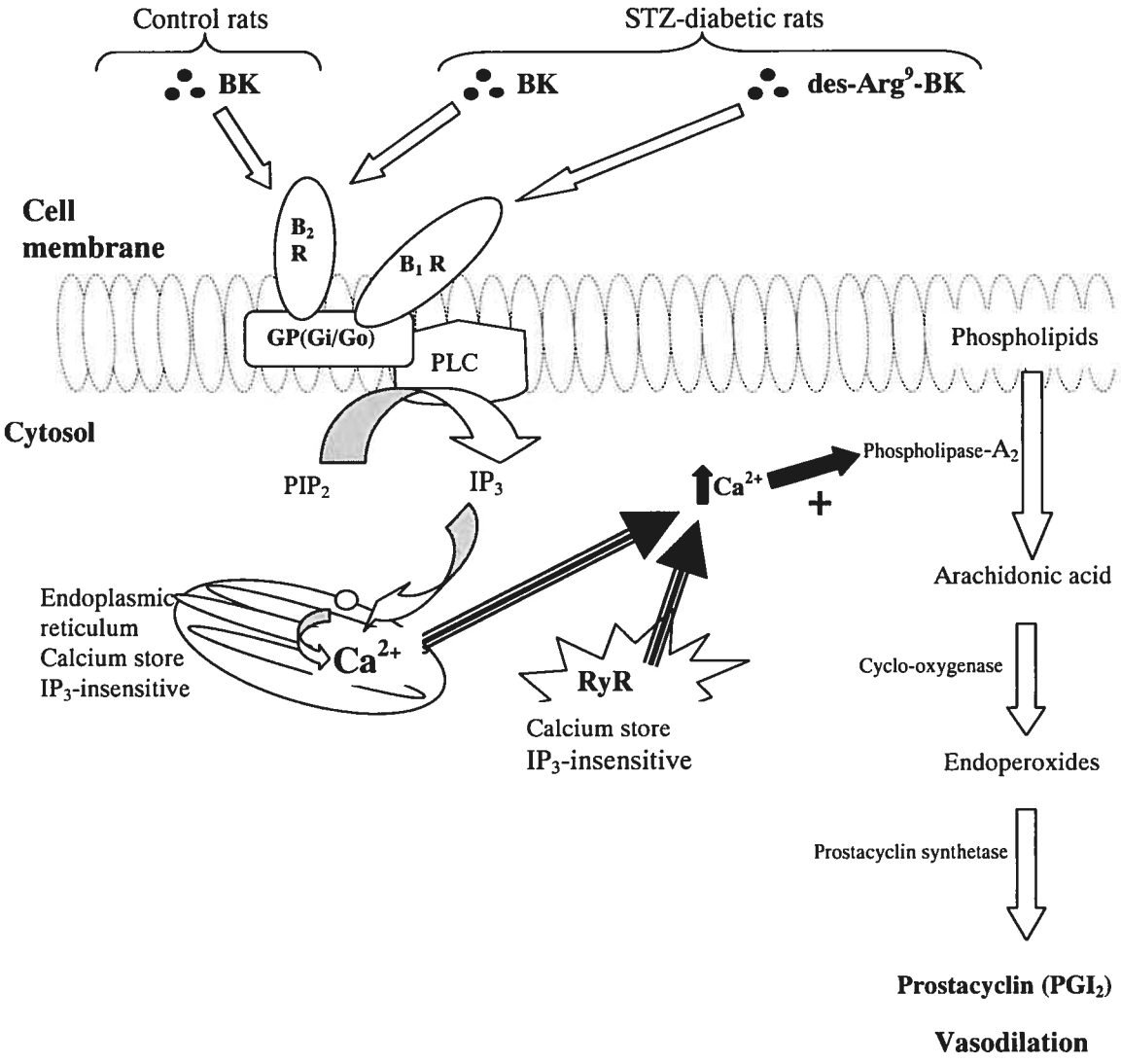


FIGURE 36. Summary of the effects of kinins on retinal vessel. The intra retinal signal pathways which are involved by the kallikrein-kinin system-induced retinal vessel vasodilation in control and STZ-diabetic rats. BK, Bradykinin; des-Arg^9-BK, des-Arg^9-bradykinin; R , kinin Receptors; GP, G-Protein; PLC, protein lipase C; PIP_2, Phosphatidylinositol 4,5-diphosphate; IP_3, Inositol triphosphate; RyR, Ryanodine receptor.

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